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PHYSIOLOGICAL REVIEWS

VOL. 27 OCTOBER, 1947 No. 4

BODY SIZE AND METABOLIC RATE

MAX KLEIBER

Division of Animal Husbandry, College of Agriculture, University of California, Davis

CORRELATION BETWEEN BODY SIZE AND METABOLIC RATE. Günther (1944) introduces a recent review on body weight and metabolic rate with a motto which starts as follows:

"It is believed that far greater progress will be made by discarding all thoughts of a uniformity in heat loss and emphasizing the non-uniformity in heat production. . . ."

The sentence is a citation from Benedict's book, *Vital Energetics* (1938, p. 194).

It is rather difficult to understand how forgetting all thoughts of uniformity and emphasizing non-uniformity can stimulate a comparison of metabolic rates of large and small animals. Any comparison presupposes a common basis, and if I were convinced of the "futility of attempts to discover a unifying principle in metabolism" (Benedict, l.c., p. 178) I should not attempt to write a review on the relation of body size and metabolic rate.

The reader can be expected to spend time on this review only when he can be convinced that body size and metabolic rate are actually related. That these two variables are related is in fact common knowledge.

Does a horse produce more heat per day than a rat or do some rats produce more heat than do some horses? Almost anybody who understands what is meant by "heat production per day" will not hesitate to give the correct answer and will even be convinced that the daily rate of heat production of men or sheep is greater than that of rats, but smaller than that of horses. Thus most people (among those who understand the question) are convinced that in general the bigger homeotherms produce more heat per day than the smaller homeotherms, that, in other words, the metabolic rate of homeotherms is positively correlated to body size.

The answer to the next question: "does a horse produce more heat per day per kilogram of body weight than a rat?" requires some biological training. Most biologists, however, will not hesitate to answer that the rate of heat production per unit body weight of the big animal is less than that of the small animal.

The positive correlation between metabolic rate and body size, and the negative correlation between metabolic rate per unit weight and body size, establish two limits between which we expect to find the rate of heat production of a horse if we know the rate of heat production of a rat. We expect the metabolic rate of the horse to be somewhat between that of the rat, and that of the rat times the ratio of horse weight to rat weight, provided of course that we do not regard these two correlations as simply accidental.

If we are firmly convinced that the metabolic rate of horses, and other homeotherms of similar size, is never outside these two limits, then we admit to recognize a natural law between body size and metabolic rate. The firmness of the

conviction, or the probability of being correct, rather than the range within which we predict a result, justifies the term "law."

For the usefulness of a law, however, the accuracy of prediction is important. We may attempt to formulate the relation between body size and metabolic rate more precisely than "somewhere in the range between rate per animal and rate per unit weight."

Encouraging for such attempts is the fact that the correlation between metabolic rate and body weight is, indeed, rather high. For 26 groups of mammals, dealt with particularly in this review, the coefficient of correlation between the two variables amounts to +0.98.

This high correlation involves standardization of conditions under which metabolic rates are measured. The standard metabolic rate should be measured while the animal is in a post absorptive condition, and kept within the range of metabolically indifferent environmental temperature.

The task is to find a metabolic body size which is chosen so that the metabolic rate per unit of this body size is the same for large and small animals.

The square meter of body surface is such a unit which allows a more accurate prediction of metabolic rate than the two limits mentioned above. The surface area of animals is, however, ill defined, and relatively recent results indicate that the metabolic rate per unit surface area tends to be greater the larger the animals (Kleiber, 1932). Among the 26 groups of animals chosen for discussion in this review the metabolic rate per unit of the 2/3 power of body weight (which roughly represents surface area) has a highly significant correlation to body weight. The correlation coefficient amounts to +0.71.

Such correlation disappears when the metabolic rate is divided by the 3/4 power of body weight. The unit of the 3/4 power of body weight, $kg.^{3/4}$, is therefore a suitable unit of metabolic body size.

Fasting homeotherms under standard conditions produce daily an average of about 70 kcal. of heat per $kg.^{3/4}$ or 3 kcal. per $kg.^{3/4}$ per hour.

The unit of metabolic body size is of great interest in comparative physiology. It permits an expression of the metabolic level of an animal independent of its body size, and it makes possible the comparison of a particular metabolic level with the interspecific mean of 70 kcal. per $kg.^{3/4}$ per day.

Such a comparison, furthermore, permits for example the statement that the metabolic rate of a 70 ton whale estimated by Irving, Scholander and Grinnell (1941) is high in comparison to that of other mammals (whereas the whale's rate per kg. is only 1/10 that of a mouse). Such a comparison, made possible by the use of metabolic body size, may stimulate research to establish the conditions producing that high metabolic rate. It may lead, for example, to a comparison with the metabolic rate of Eskimos which also is at a relatively high level (Rabinowitch and Smith, 1936). The unit of metabolic body size is further useful in evaluating levels of food intake in animal production, and in classifying farm animals with regard to their efficiency as food utilizers. Food requirements and dosages of most vitamins and drugs may be expressed in terms of metabolic body size.

This review is limited to mammals. The conclusions are, however, also applicable to birds whose metabolic level was found to be in line with the mammalian metabolic level (Kleiber, 1932). More recent results confirm the metabolic similarity of these two groups of homeotherms. From data published by Dukes (1937) one may calculate that the metabolic rate of hens is 73 kcal. per kg.$^{3/4}$ A rule of body size and metabolic rate, very similar to that for mammals and birds, has also been noted in heterotherms such as the crab by Weymouth and his co-workers (1944).

THE DISCOVERY OF THE SURFACE LAW. The metabolic rate (heat production per unit of time), in particular the basal metabolic rate of humans, is generally expressed in kilocalories per square meter of body surface. This procedure is based on the theory that in animals of different body size the metabolic rate is proportional to their respective surface areas. This theory, called the surface law, is now a little over a century old. It has its roots in the time when a vigorous belief in the power of reason inspired men to explain nature's aims and means. A good deal of a new theology was then evident in teleological scientific discussions. A theology in which Nature, as a bright goddess, revealed her aims and ways to a scientist as a more stern and sombre Lord had revealed His aims and wishes to the nonscientific or antiscientific priests.

Sarrus, a professor of mathematics, and Rameaux, a doctor of medicine and of science, both in Strasbourg, had Thillaye read a thesis to the Royal Academy of France during its meeting of July 23, 1839.

The authors apparently take it for granted that it is nature's aim to make the rate of heat production of large and small animals in proportion to their respective surface areas or the 2/3 power of their body weights. They then calculate how nature achieves this aim by modulating the frequency of heart beat and the stroke volume, always choosing the middle way between two possibilities. Their basic theory is worth being quoted: (p. 1098) "Lorsque la nature peut attaindre un but par plusieurs moyens, elle n'emploie jamais exclusivement l'un d'eux jusqu'aux limites, elle les fait concourir de manière que chacun de ces moyens tend à produire une part égale de l'effet total."

(When nature can achieve an aim by various means she never uses one of these means exclusively to the limit, she makes these means compete so that each one of them produces an equal part of the total effect.)

Bergmann and Leuckart (1855) concluded from measurements of Regnault and Reiset that the metabolic rate per unit weight was especially great for small animals. In four days sparrows consumed as much oxygen as they weighed. This today would be considered a very high metabolic rate, about four times as high as the rate observed in a sparrow by Benedict (1938). Richet (1889) discovered "après coup," that is empirically, that the metabolic rate per unit weight of rabbits increased consistently as the body weight decreased (p. 220).

The metabolic rate per unit surface area, however, was unaffected by body size, so Richet concluded that, for metabolic rate, surface area was more important than body weight. Simultaneously Rubner (1883 cit. by Krogh 1916, p. 133) noted a systematic decrease of the metabolic rate per unit weight of

fasting dogs as the weight increased from little dogs of 3 kg. to big specimens of 31 kg. body weight.

When the metabolic rate was expressed per square meter of body surface, however, the effect of body size disappeared. From this and similar observations, Rubner deduced his simple rule that fasting homeotherms produce daily 1000 kcal. of heat per square meter of body surface.

Richet's and Rubner's intraspecific observation of the surface law was confirmed by interspecific comparisons. The best known table showing this surface law of metabolic rate is that published by Voit (1901). A 441 kg. horse produces over 948 kcal. daily per square meter of body surface, a 64 kg. man 1042, a 15 kg. dog 1039, and a 2 kg. hen 1008. So well established appeared the surface law that data which did not confirm it were either explained by particular conditions[1] or discarded as results of faulty measurements. Thus Lee (1929) suggests that Mitchell's rat surface is too high because it makes rats disobey the surface law.

THE "TRUE" BODY SURFACE AREA. Large and small bodies of similar shape have surface areas in proportion to the squares of their linear dimensions or the 2/3 power of their volumes. If the two bodies have also the same density, then their surface areas are also in proportion to the 2/3 power of their weights.

In this general way Sarrus and Rameaux understood the surface area of animals. Richet apparently preferred a definite surface that could be visualized. He calculated the surface area of his rabbits from their weights assuming they were spheres with a density of 1 kg. per liter. The surface area of such a sphere is 4.84 $W^{2/3}$ square decimeters when W is the weight in kilograms.

Considering that the animals are really not spheres, Meeh (1879), working under Vierordt, substituted in Richet's formula for the factor 4.84 a term, k, which is constant only within a group of similarly shaped animals but differs according to the shape of the animal.

By measuring animal surfaces and weights one may determine the Meeh constant empirically and then calculate the actual surface of similar animals as $S = K \cdot W^{2/3}$, where W is the body weight in kilograms and S the surface area in square decimeters. A set of Meeh constants is given by Lusk (1928, p. 123).

The surface of man averages 12.3 square decimeters per unit of the 2/3 power of body weight (kg.$^{2/3}$). For slim people the Meeh constant would be higher than this average, for stout people lower. Du Bois and Du Bois (1916) developed a formula which allows calculating the actual surface area of stout and slim human beings more accurately than does the Meeh formula.

Du Bois' formula reads:

$$S = 71.84\ W^{0.425} \cdot L^{0.725}$$

S = Surface area in cm.^{2}
W = body weight in kg.
L = body length in cm.

It is probably the best method of estimating the "actual" surface area of man. Du Bois' formula is dimensionally correct and is therefore valid for any size,

[1] Rabbits produced daily only 776 kcal. of heat per square meter; subtraction of their ear surface, however, brought their metabolic rate up to the more legal level of 917 kcal.

as it should be. (Dimensionally correct means that the surface area is expressed in terms of the 2/3 power of the volume or in the square of the linear dimension.)

Since for similar bodies L is proportional to the mean linear dimension, $W^{1/3}$, Du Bois' formula for comparison of large and small humans of the same relative stature may be written as

$S = k_1 W^{0.425} \cdot k_2 W^{1/3 \times 0.725}$ which amounts to $k_1 \cdot k_2 \cdot W^{(0.425 + 0.242)}$ or $k_1 k_2 \cdot W^{2/3}$

Some later formulas for the calculation of surface area from the weight are dimensionally incorrect, such as the Moulton formula for cattle in which the surface area is proportional to the 5/8 power of body weight, and particularly the formula used by Ritzman and Benedict (1931) for calculating the surface area of sheep as proportional to the 0.561 power of body weight. The formula may have expressed the surface area of the sheep used for the measurements but it is theoretically incorrect for two animals of similar form which differ in size (but have the same surface per kg.$^{2/3}$). The Ritzman-Benedict formula would predict too small a surface for the larger of two animals with similar build.

Many methods have been invented for measuring the surface area of animals.

In their eagerness to refine the surface measurements, many workers in this field seem to have overlooked a major question: "What is meant by the surface area?" Unless this question can be answered definitely, how can one decide which of two methods measures the surface more accurately?

One should obviously know whether or not the "true" surface of a rabbit is to include the surface area of the rabbit ears (see Benedict, 1934). As long as this question is open, which means an uncertainty of about 20 per cent, what is gained by refining the surface measurements to an accuracy of one per cent? According to Lee's (1929) (see p. 514) argument, the true body surface is the one that makes an animal's metabolic rate obey the surface law!

Kleiber (1932) pointed out that the various refinements of surface measurements and calculations, instead of clarifying issues, led to a more and more chaotic situation.

A great many published results of good work on metabolic rates are practically lost for comparative physiology because they are expressed only per unit of surface area, and the authors did not furnish the data which would make a comparison with other work possible. It was stated that the surface area was not well enough defined to serve as a basis for measurement, and, following Krogh's (1916) suggestion, and in agreement with Stoeltzner (1928) and Brody, Comfort and Matthews (1928), a power function of body weight was suggested as the basis of metabolic body size. The 3/4 power was proposed as the best fitting function (Kleiber, 1932).

Since then the situation has not improved. In the Annual Review of Physiology (Kleiber, 1944) alarm is again expressed as follows:

"In 10 papers (from 8 laboratories) studied for this review metabolic rates of rats are expressed per unit of the surface area. Four of the 10 authors did not state how they measured or calculated this area. One multiplied the 2/3 power of body weight (in kg.) by 7.42, another by 9.1, a third by 10, to calculate the

surface area in square decimeters. One author multiplied the 3/5 power of body weight by 12.44, and two have calculated a new surface-weight relationship, presumably by an intricate logarithmic interpolation between three older ones $7.42 \times W^{2/3}$, $11.36\ W^{2/3}$ and $12.44\ W^{3/5}$. That—for rat metabolism only—is this year's result of a century of surface law."

For man, the formula of Du Bois seems to be so generally accepted that the reports do not differ as widely as do those of rats, and clinicians may not have the time to develop new surface formulas for every new paper they publish. But also with human metabolism the valuable fruit of years of tremendous work cannot be used properly for comparative physiology, because the results are reported only in terms of the Du Bois surface without the data (height and weight) which would make a recalculation of the original results at least possible, even if extremely time consuming.

The comparisons of metabolic rates merely on the basis of surface area may be all a clinician wants. For comparative physiology more basic data are necessary.

In the interest of economy in research, physiologists should agree on minimum requirements for publication of metabolic data, and among those should be body weight and either total metabolic rate or metabolic rate per unit weight.

THEORETICAL VALIDITY OF THE SURFACE LAW. *A. The various theories of surface law.* The theories advanced for the interpretation of the surface law of animal metabolism may be classified into 5 major groups:

The metabolic rate of animals must be in proportion to their body surface.

1. Because the rate of *heat transfer* between animal and environment is proportional to the body surface area.

2. Because the intensity of flow of nutrients, in particular oxidizable material and oxygen, is a function of the sum of *internal surfaces* which in turn is proportional to the body surface.

3. Because the rate of supply of oxidizable material and oxygen to the tissues is a function of the mean intensity of the *blood current*, which is proportional to the square area of the blood vessels, which in turn is proportional to the area of body surface.

4. Because the *composition* of the animals is a function of their body size. The composition may be meant either *anatomically*: the larger the animal the lower is the ratio of the mass of metabolically active organs to the mass of metabolically inert organs; or the composition may be meant *chemically*: the larger the animal the lower its percentage of "active protoplasm".

5. Because the cells of the body have an *inherent requirement* of oxygen consumption per unit weight, which is smaller the larger the animal.

B. Critique of the theories of surface law. 1. The *heat transfer theory* is the most convincing of the five interpretations of the surface law. If homeothermism has survival value, and if the rate of heat transfer per unit surface area of large and small bodies is equal, then the surface law may be understood as the result of natural selection.

Accepting for the sake of the present discussion the biological advantages of maintaining a constant body temperature, one may ask: "How accurate is the

postulate that the rate of heat transfer per unit of surface area of large and small bodies is the same?"

The rate of heat flow of a body covered by an insulating layer may be formulated according to Fourier (Mach 1919, p. 84):

$$q = S\lambda \frac{T_i - T_o}{L}$$

where q = rate of heat flow; S = surface area
L = thickness of the covering layer (skin and fur)
T_i = internal temperature
T_o = temperature at the surface
λ = heat conductivity.

In animals the heat conductivity, λ, of the body covering, and its thermoconductive thickness, L, are rather hard to measure and are, furthermore, variable, since the depth of the layer with a temperature gradient is under vasomotor control. To simplify the problem, one may define the ratio $L/\lambda = r$ as the specific insulation of the animal covering, and write Fourier's law as follows:

$$q = S \frac{T_i - T_o}{r}$$

For a given difference between internal temperature and surface temperature, the rate of heat transfer is proportional to the surface area when the specific insulation for large and small bodies is the same. The specific insulation of animals is, however, variable.

The classical demonstration of this fact is the experiment of Hoesslin (1888). He reared two littermate dogs, one at 32°, the other at 5°C. The latter had to cope with a temperature difference between body and environment six times as great as the corresponding difference for his brother. Yet the metabolic rate of the dog in the cold was only 12 per cent higher. He solved the problem of keeping warm by growing a fur that weighed three times as much as that of his brother.

In the same animal and within a short time, the specific insulation may be reduced to 1/2 when the animal is transferred from a cold to a warm environment (Kleiber, 1932). Instead of maintaining the metabolic rate per unit surface area constant, large and small animals therefore might maintain a constant metabolic rate per unit weight, and with a variable specific insulation adapt the rate of heat loss to that metabolic rate.

The range within which the specific insulation can be changed is, however, limited. One may calculate (l.c., p. 327) that a 60 gram mouse with the same metabolic rate per unit weight as a steer, would need the equivalent of a steer's surface covering in a 20 cm. thick layer to maintain its body temperature in an environment of 3°C. This rather extreme example illustrates why it is advantageous for a small animal to have a higher metabolic rate per unit weight than a large animal. One can similarly show why at a high environmental temperature, for the prevention of overheating, it is advantageous for a large animal to have a lower metabolic rate per unit weight than a small animal.

Considerations of heat transfer thus make the assumption reasonable that natural selection would weed out such representatives of cattle and rats that insisted on having the same metabolic rate per unit body weight. These considerations however do in no way support the hypothesis that the metabolic rate of animals should be strictly proportional to their exact body surface, and that the results of metabolism studies would be more accurate and more reliable the more accurately one measured the "true" surface area of the animals.

2. The theory of *internal surfaces* as interpretation of the surface law would be valid only if the size of cells (or alveoli in the lungs as recently referred to again by Gaija, 1946) were proportional to animal size, if, in other words, elephants were made up of the same number of cells as mice. This logical prerequisite for the theory of internal surfaces does not seem to be supported by histology.

3. Attempts have been made to derive an interpretation of the surface law on the basis of *blood circulation*. The difference in the oxygen content of arterial and venous blood is independent of body size. The rate of oxygen consumption is therefore proportional to the intensity of the blood current, as already postulated by Sarrus and Rameaux (1839). This current intensity is the product of the linear velocity and the cross section area of the duct. Hoesslin (1888) declared that the cross section area of the aorta is proportional to the 2/3 power of body weight and the linear velocity is independent of body size, that consequently the intensity of the blood current is proportional to the 2/3 power of body weight, which is a measure for body surface. That the square area of the aorta should be proportional to the 2/3 power of body weight, appears sound application of the principle of similarity. Why, on the other hand, the same linear velocity of blood flow in the aorta for large and small animals should have particular survival value is not quite clear. (See Kleiber, 1932, p. 332.)

More convincing support of a circulatory interpretation of the surface law is gained by postulating, on the basis of the similarity principle, that heart volume or stroke volume of large and small animals should be approximately in proportion to their body weights.[2] On mechanical grounds the assumption seems reasonable that the heart of a horse cannot beat with the frequency of the heart of a mouse. The intensity of the blood current (stroke volume times frequency of heart beat), therefore, should increase less than in direct proportion to increase in weight.

$I = f \cdot v$, where I = intensity of blood current, f = frequency of heart beat and v = blood volume per heart beat. If the stroke volume is proportional to body weight, $v = kW$ then, $I = f \cdot kW$, and the Intensity per unit weight $\frac{I}{W} = kf$.

To explain the Surface law, the intensity of the blood current should be proportional to the 2/3 power of body weight, and, therefore, the frequency of heart beat should be inversely proportional to the cube root of body weight. ($f = k_1\frac{I}{W} = k_2\frac{W^{2/3}}{W} = k_2W^{-1/3}$).

From data given by Rihl (1927) for normal pulse frequency of various animals ranging from

[2] The similarity is actually not quite strict. Clark (1927, p. 72) concludes from interspecific comparison that heart weight varies with the 0.9 power of body weight. The heart weight per unit body weight has thus the tendency to become smaller the larger the animal.

rabbits to elephants, one may deduce the rule that the pulse frequency is inversely proportional to the 4th root of body weight. (The pulse frequency is on the average 186 $W^{-1/4}$ if W is given in kg.)

Considering heart volume and pulse frequency, one may therefore be satisfied that, from the point of view of oxygen transport, it is advantageous for large animals to have a lower metabolic rate per unit weight than small animals. Obviously, this consideration does not lend support to the hypothesis that the metabolic rate should be strictly proportional to the "true" body surface.

4. There appears to be very little evidence in support of the idea that the *chemical composition* of animals changes systematically with body size, so that the surface law can be understood on the basis of chemical composition. The concept of "metabolically active protoplasm" seems to be too vague for discussion. It seems that the activity of the protoplasm is determined by the metabolic rate; and what would then be gained by explaining, in turn, the metabolic rate on the basis of active protoplasm? The measurement of the nucleo-protoplasmic mass seems to offer an independent determination of active protoplasm, but Lindeman (1943) failed to find a correlation between nucleo-protoplasmic mass and rate of oxygen consumption of the retina.

The concentration of respiratory enzymes, especially cytochrome *c*, may be related to metabolic rate (Rosenthal and Drabkin, 1943), however this concentration itself, and particularly the activity of the enzymes, probably is under the influence of regulators, especially the endocrine system. (See Barron, 1943.) Enzyme concentration and enzyme activity may thus offer a valid explanation for the metabolic rate of tissues but in regard to body size and metabolic rate, enzyme concentrations and activity call for further explanation on the organismic level.

A good deal of speculation has centered around the anatomical composition as an explanation of the surface law.

Dreyer, Ray, and Walker (1910, p. 158) advanced the theory that the blood volume of animals was proportional to their surface areas. On the basis of the very data from which these authors draw this conclusion, one may demonstrate that, in general, the blood volume is more nearly proportional to body weight than to body surface (Kleiber, 1932, p. 329).

In direct opposition to the conclusion of Dreyer, Ray and Walker, furthermore, is a later observation by Gibson, Kelley and Pijoan (1938), who noted that in a series of dogs the blood volume per kg. body weight increased from 84 cc./kg. in 5 kg. dogs, to 97 cc./kg. in 30 kg. dogs.

Sweeping conclusions, on the basis of measurements on limited material, are often misleading.

Brody, Comfort and Mathews (1928, p. 33) made the following statement: "The weight of the kidney, the weight of the liver and practically the weight of the lung, blood, stomach, and intestine increase directly with the body weight at the same relative rate as does the surface."

This statement presumably led to the following sentence by Benedict (1938, p. 205):

"Our conclusion is, therefore, that if the brain weight is closely related to the heat production among animals other than the primates, this is in large part due simply to the general morphological development in proportion to the two thirds power of the body weight shown by innumerable body structures."

Before such generalizations are announced, they should be tested rationally by application to extreme cases. This can be done with the allegation that the blood volume is approximately proportional to body surface.

According to Lindhard (1926), the amount of blood in man is about 5 per cent of the body weight. If the statement of Brody et al. or Dreyer et al. were generally correct one would have to conclude from the blood content of a 70 kg. man that 49 per cent of the body of a 70 g. rat would be blood—indeed a bloody rat!

The following calculation similarly illustrates that one should be cautious when one derives a general quantitative rule from the tendency, that large animals have relatively smaller brains than the small animals.

A 5.4 ton elephant had a brain of 7.5 kg. (Benedict, 1936). The brain weight amounted, thus, to 0.14 per cent of the body weight. If the brain weight (in non primates) were proportional to the 2/3 power of body weight, the brain of a 5.4 gram shrew would amount to 14 per cent of its body weight, or that of a 100 gram rat to 5 per cent of its body weight, which is about 3 times as much as it actually weighs (Donaldson, 1924).

There seems to be a general tendency for the larger animals to economize on brain weights, as J. B. S. Haldane (1946) pointed out in his stimulating essay ("On being the right size"), but what he observes within a group of Felidae from cat to tiger can not be regarded as a general quantitative rule. If generally the weight of the brain were only doubled whenever the body weight is quadrupled, or in other words, if the brain weight were proportional to the square root of body weight, then, starting down from the elephant with a brain weight of 0.14 per cent of its body, one would conclude that one-third of a 100 gram rat should be brain. Similarly, if Dubois' (cit. by Weber, 1923, p. 151) formula, making brain weight a function of the 5/9 power of body weight, were generalized, the 100 gram rat would have to have a brain weight of 18 grams. Neither Dubois nor Haldane can be accused of generalizing the brain weight-body weight relationship so much as to include the comparison of rats and elephants. Such generalization is, however, made when this relation of brain weight to body weight is used to explain the surface law of metabolic rate, because that law includes comparisons of rats with elephants.

The idea that the surface law of metabolism can be explained by the anatomical composition of the animal has been advanced especially by Blank (1934), and by Kestner (1934 and 1936) who made the following statement. "Hence I conclude that the relative sizes of the brains and the large glands can give a complete explanation of the different heights of metabolism of different animals."

In support of this idea Kestner gives the ratios of brain weight to body weight of large and small dogs. A dog of 40 kg, for example, weighs 400 times as much as its brain. A dog of 5 kg, however, weighs only 25 times as much as its brain.

By calculating from these data the absolute weight of the brains we would be led to the somewhat surprising result that the brain of the small dog weighs 200 grams; that of the large dog only 100 grams.

To support his point that higher metabolic rates of the smaller animals are explainable by their greater relative brain size, Kestner states that the brain weight of a 3 kg. dog is about 50–60 grams whereas that of a rabbit of the same body weight is only 10 grams. This comparison, however, far from supporting Kestner's theory that the brain weight dominates the metabolic rate, to the contrary is very good evidence against this theory. Despite the great differences in relative brain weight noted by Kestner, the mean metabolic rate of rabbits is only about 10 per cent lower than that of dogs of the same size (see table 1).

It is generally accepted that primates have exceptionally large brains. If brain weight dominated metabolic level, primates should have a particularly high metabolic rate. Yet a group of college women with an average body weight of 54.8 kg. (and an average brain weight of probably not much less than 1300

TABLE 1

	BODY WEIGHT	DAILY HEAT PRODUCTION PER SQUARE METER	
		1901	1931
	kg.	*kcal*	*kcal*
Horse	441	>948	
Steer	342		1465
Man	64	1042	926
Dog	15	1039	776
Hen	2	1008	676

grams) had an average basal metabolic rate of 1224 kcal. per day, which is slightly less than the corresponding average of 1254 kcal. per day for a group of female sheep with an average weight of 46.4 kg. and a brain weight of, presumably, about 100 grams (Pálsson 1940).

5. *Body size and tissue metabolism* is a problem that is not yet satisfactorily solved. In the paper in which Rubner (1883) demonstrated the surface law in the metabolic rate of dogs he wrote (p. 550):

"Large and small dogs have a different metabolic rate, not because there are definite differences in the organization of their cells, but because the impulses originating in the skin from cooling stimulate the cells to metabolic activity."

The sum of these impulses, according to Rubner, is in proportion to the body surface.

Later on Rubner accepted just the opposite view, declaring that the metabolic rate of the tissues is a fundamental characteristic of a species (Wels, 1925).

In 1925 Terroine and Roche derived from microrespiration trials with excised animal tissue a hypothesis opposed to Rubner's idea of genetically fixed cell metabolism. They stated: "Homologous tissues of different animals have, in vitro, the same intensity of respiration." The metabolic differences of tissues

of large and small animals disappear when the tissues are removed from the animal body. These differences, therefore, are not located in the cells themselves, but are imposed on the cells by central systems representing the organism as a whole, the nervous system and the circulatory system.

In the same year Grafe (1925), independent of Terroine and Roche, reached the same conclusion stating: "The living protoplasm of the warm blooded animals and maybe even of many cold blooded animals, shows, as far as respiration is concerned, a certain uniformity, and gets its specificity only by means of the regulating system of the animal!"

The tissues, according to Grafe, respire at a higher rate in vitro than in vivo, particularly tissues of large animals. In vivo the metabolic rate of those tissues is checked by the influence of the central regulators, mainly the nervous and endocrine systems.

There are a number of observations in support of this view. Severance of the spinal cord increased the rate of nitrogen metabolism (Isenschmid, 1920). In line with these ideas is also the observation of Victor (1934) that dystrophic muscles have increased metabolic rate in vitro. Recently Houchin (1942) stated that normally tocopheryl phosphate acts as a brake on muscle oxidation, and that vitamin E deficiency lets the oxidation "run riot" so that the muscle is "consumed in its own fire" and muscle dystrophy results.

The discovery of capillary regulation of blood supply to the tissues by *Krogh* (1929) led to a well rounded theory that the checking of metabolic rate in vivo was accomplished by rationing of the oxygen supply. That was in contrast to Pflüger's law (Pflüger, 1872), to be sure, but observations of Thunberg (1905) on lower animals, and of *Verzár* (1912) on muscles, indicated that Pflüger's law loses its application at low partial oxygen tensions and that oxygen supply may become the limiting factor in the rate of tissue respiration. Recently Gaija (1946) noted that below a given critical partial pressure of oxygen the metabolic rate of homeotherms depends on oxygen supply. Apparently, independent of body size, the limiting partial oxygen pressure amounts to about 90 mm. Hg. This corresponds to a barometric pressure of 450 mm. Hg. or an altitude of 4000 meters.

That the metabolic rate of animals was by no means a genetically fixed constant, but was decidedly affected by somatogenic factors, was demonstrated by Kleiber and Cole (1939) who measured the metabolic rate of giant rats produced by injection of growth hormone. The metabolic rate, either per unit weight or per unit of the 2/3 power of weight, was lower in the growth hormone rats than in normal litter mate controls. This rate could therefore not be a genetically fixed characteristic of the tissues (being changed by the somatic influence of growth hormone injection). This result was in line with the ideas of Terroine and of Grafe. Kleiber and Cole, however, observed also that the differences in the metabolic rates of giant growth hormone and normal control rats could still be noted in the rate of oxygen consumption of the surviving diaphragm in vitro. This observation was in opposition to the results of Terroine and of Grafe.

Field, Belding and Martin (1939) succeeded in summating the metabolic

rates of excised rat tissues in vitro to 66 per cent of the actually measured metabolic rate of the rat. Martin and Fuhrman (1941) similarly summated metabolic rates in vitro of dog tissues to over 70 per cent of the metabolic rate of the dogs. These results support the idea that the rate of oxygen consumption of the tissues in vitro reflects the metabolic rate of these tissues in vivo to a considerable extent.

Repeating the investigations of Terroine and of Grafe, Kleiber (1941) measured the in vitro rate of oxygen consumption of liver slices from rats, rabbits, and sheep. The metabolic rate per unit weight in vitro decreased consistently with increasing size of the animals, almost to the same extent as the metabolic rate of the living animal.

Recent microrespiration trials in our laboratory (Kleiber, 1947, unpublished) with excised liver of rabbits and cows again show that liver slices from cows have a lower rate of oxygen consumption per unit weight than the liver slices from rabbits.

Smyth (1940), on the other hand, reports that "observations by Van Heyningen (1936) indicate a uniformity in the rate of metabolism in the same tissue in different species irrespective of the size of the animal."

This result seems to be in line with the theory of Grafe and of Terroine and in contrast to our findings. A check of Van Heyningen's figures, however, indicates that only anaerobic glycolysis of brain cortex was independent of body size. For respiration, the effect of body size on tissue metabolism was similar to our results.

The following working hypothesis seems justified: The animal as a whole responds to changes in somatic conditions. Reactions to such changes are transmitted to the tissues by centralized metabolic regulators such as the nervous and the endocrine systems. These regulatory influences produce changes in the metabolic conditions of the cells. Among these conditions, which determine cellular metabolic rate, may be structural relations, electrical potentials, ion concentrations, and the concentrations of oxygen, metabolites and enzymes.

The observation that metabolic rates in vitro are similar to those in vivo suggests that the conditions which determine the rate of cell respiration change but slowly; this would be expected of changes in concentrations of enzymes and metabolites.

Since oxygen supply should not affect the oxidation rate in vitro, the similarity between in vitro and in vivo metabolic rate throws doubt on the theory that tissue metabolism normally is regulated by rationing the oxygen supply.

The observation that the metabolic rate of tissues in vitro is similar to the corresponding rate in vivo, is sometimes interpreted to indicate an inherent metabolic level of the tissues. If the statement means that the rate of tissue respiration is independent of somatic influences, then it is wrong; if it means that genetic factors are involved in the metabolic levels, then the correctness of the statement is hardly in doubt. One may be able to breed strains of rats with a high, and strains of rats with a low metabolic level under quasi equal environmental conditions. There may be genetically determined limitations

for the extent to which cell metabolism reacts on changes produced in the cells by the metabolic regulators. The demonstration of genetic factors in metabolic level, however, helps but little in the interpretation of the fact that the metabolic rate per unit weight of a 5.3 kg. rabbit is practically the same as that of a 6.6 kg. dog, but differs significantly from that of a 1.5 kg. rabbit; as in turn the metabolic rate per unit weight of a 25 kg. dog differs significantly from that of the 6.6 kg. dog.

The results of Terroine and of Grafe on metabolic rates in vitro were not confirmed later, yet an essential part of their theory is sound; namely, the idea that the metabolic rate of the tissues in vivo is controlled by central regulators representing the animal as a whole.

Even fetal metabolic rate seems to be under the influence of the maternal metabolic regulators (or perhaps a combination of maternal and fetal regulators).

Kleiber, Cole and Smith (1943) measured the metabolic rate of rat embryos in vitro, and obtained good evidence against the theory that a fetus behaves, metabolically, like an independent small homeotherm. The fetal metabolic rate per unit moist weight was of the same order of magnitude as that of normal adult rats, and considerably smaller than that of newly born or 12 day old rats.

This observation, like others mentioned, favors the idea that, to a considerable degree, cellular metabolic rate is adapted to the condition of the animal as a whole. The effect of the metabolic regulators, however, appears to remain in the tissues that are removed from the animal and respire in vitro.

The analysis of these factors controlling the metabolic level in vitro and in vivo appears to be a most fruitful field for future research on tissue metabolism.

C. Integration of valid theories on surface law. Of the five groups of interpretations of the surface law, the one on internal nutritive surfaces has no evidence in its favor. The one on composition of the body has no explanatory value. The same is true for the explanation with a genetically fixed metabolic rate of body cells, even if this postulate as such were acceptable, because the problem would arise: why have those animals been selected for survival whose cells have the inherent metabolic rate such that the metabolic rate follows the surface law?

The theories that relate the surface law to rate of heat transfer, and those that relate it to the hemodynamics, have most value for the interpretation of the surface law. The most promising theory of body size and metabolism integrates the heat exchange and the circulation theory.

In natural selection, those animals probably prove to be the fittest whose cells are adapted to such a level of oxygen consumption that the metabolic rate of the animal is most suitable for the maintenance of a constant body temperature and in line with the most efficient transport of oxygen.

A four ton animal, whose cells insisted on a rate of oxygen consumption per unit weight equal to that of mouse cells, could not survive, because such a metabolic rate could not be supported by the circulatory system and would upset the maintenance of a constant body temperature.

The surface law is unreliable mainly because the definition of an animal's surface is vague. But even if the surface area could be defined and measured

accurately there is no theoretical basis for the hypothesis that the metabolic rate of homeotherms should be exactly proportional to their particular surface area rather than to a more general function of body size.

EMPIRICAL VALIDITY OF SURFACE LAW. Between the efforts of the gadgeteers to design apparatuses for surface measurements, the statisticians to derive formulas for calculating "true" surface areas, and the theoretically inclined biologists who discussed the proper interpretations of the surface law, rather few seemed to have been interested in the question as to just how reliable the surface law itself is.

Among the few that questioned rather early the validity of the surface law was one of the pioneers of vitamin research, F. G. Hopkins, who in 1912 wrote as follows:

"... in the case of very young rats the demand for maintenance is more nearly determined by the live-weight than by the surface area. It becomes of course smaller relatively to body weight as this increases but it falls off more slowly than the surface area would require."

Seven years later, Harris and Benedict (1919) concluded from their extensive measurements on men and women that within the human species there was no evidence for the surface law. Du Bois (1927, p. 202) however, maintained that the data of Harris and Benedict confirmed the surface law.

The differences in size among the material of Harris and Benedict were so small and the influence of factors other than size so relatively large that the two opposing deductions could both be made in good faith (compare pp. 530 and 534).

In an attempt to find the most suitable unit of metabolic body size for estimating maintenance requirements and comparing metabolic rates of animals that differ in weight, Kleiber (1932) compiled results of metabolism measurements from American laboratories, listing 13 groups from 150 gram ring doves to a 679 kg. steer. The surface law was confirmed insofar as the metabolic rate per unit weight decreased systematically as the body weight of the animals increased. The coefficient of variability per unit weight was 80 per cent, and that per unit surface area only 34 per cent. This latter is still quite high, and a comparison of some of the recent results with Voit's table (1901) apparently indicates a trend of the modern American animals to take the surface law less seriously than the European animals in 1901.

There is a considerable positive correlation between body size and metabolic rate per square meter of body surface.

The surface law, that is the theory that the metabolic rate per unit surface area of large and small animals is the same—or at least independent of size—is therefore not strictly confirmed by the recent material.

METABOLIC RATE AS A POWER FUNCTION OF BODY WEIGHT. *A. Linear relation between logarithms of metabolic rate and body weight.* The regularity in the deviations of the empirical results from the surface law justified the search for a function of body size to which metabolic rate might be more nearly proportional than to body surface. Plotting the logarithms of fasting metabolic rate against the logarithms of body weight revealed a linear relation between these two variables

with surprisingly small deviations from the mean trend. For the ten groups of mammals, the standard deviation from the mean regression line amounts to ±0.03 logarithm unit, which corresponds to a coefficient of variation of metabolic rate from the interspecific mean of 7 per cent.

When the logarithm of metabolic rate is a linear function of the logarithm of body weight, then metabolic rate is proportional to a given power of body weight.[3] The metabolic rate was more nearly proportional to the 3/4 power of body weight than to either the 2/3rd power or the surface area of the animals (as derived for each group by a special formula designed for the calculation of its particular surface area).

Soon after the publication of these results, Brody and Procter (1932) extended a similar compilation of metabolic data down to the mouse. They used mostly figures accumulated in their own laboratory and noted that their result was in close agreement with that of Kleiber (l.c., p. 94).

In 1938, F. G. Benedict published an extensive analysis of the great amount of valuable data on metabolic rates of various animals tested in the Carnegie Nutrition Laboratory by reliable methods under rather well standardized conditions.

A chart (p. 171) shows the logarithms of the mean metabolic rates for 28 groups of animals plotted against the logarithms of the corresponding mean body weights. The animals considered ranged in size from 20 gram mice to nearly 4 ton elephants. A regression line indicates the average trend, and Benedict notes "a most gratifying straight line relationship between the total heat production and the body weight." He obviously means the logarithms of these variables. He then continues as follows: "However satisfactory this relationship may be mathematically, this method of presenting the data completely masks metabolic differences within species."

In answer to this remark one may say that any mean of a group of data "masks," that is, does not show, the differences between the single data; that has nothing to do with logarithmic interpolation. If one wants to show the differences within the species one may plot the logarithm of each individual metabolic rate against logarithm of the corresponding individual body weight. In order to supply the reader with an estimate of the variability, as a substitute for presenting every single result, some of the more statistically minded biologists state with a mean also its standard error.

Benedict extends his accusation, stating that logarithmic interpolation "distorts or obscures striking differences between the species." Since, however, a logarithmic chart in a scientific paper is presented to readers who are presumably familiar with logarithms, the accusation of distorting or obscuring can be discarded.

There is nothing obscure about the fact that a logarithmic regression line of a given set of data looks different, in general, from the corresponding arithmetic line, and if this difference in the appearance of the two regression lines be termed

[3] If $\log M = \log a + p \cdot \log W$—(general equation for straight line)
then $M = a \cdot W^p$

distortion, one could call the arithmetic line a distortion of the logarithmic just as well as vice versa.

Benedict further believes (p. 172) that "the seeming similarity between the different species shown by this logarithmic chart is an artificial similarity."

It is true that the conditions under which basal metabolism is measured are somewhat different from those under which animals normally live. The conditions under which the results discussed here are obtained may therefore be classified as artificial, and it might be argued that the metabolic rate of a rat in a sewer, or a cow in a barn, would be different from their metabolic rates measured in the artificial environment of a respiration apparatus; but that possibility has nothing to do with the question, whether or not the linearity of the logarithms of metabolic rates as measured and the corresponding body weights are real. Whether or not such a relationship has physiological significance depends on the physiologist, the same as it depends on the listener whether he hears a symphony or merely a multitude of different sounds.

Benedict concludes his chapter on interspecific comparisons (p. 179) with the following sentence:

"It seems, therefore, unjustifiable to apply mathematics to the pooled end result of the activities of millions of cells each highly differentiated, with different energy potentialities and actuated by different stimuli."

If this is the way Benedict feels, one cannot help but wonder how he ever became interested in conducting a respiration trial and why, furthermore, he even calculated means of groups of several of these pooled end results which indeed is applying mathematics.

Admittedly one may find biological publications in which data seem to be used merely as material for mathematical exercises. Admittedly, furthermore, some biologists learn mathematical tricks and tirelessly apply them, apparently without bothering to understand either the basis and limitations of their methods or the meaning of their results. Publications originating under such circumstances may have developed Benedict's antagonism to the application of mathematics in biology. The answer to such pseudomathematics in biology, however, is not less mathematics, but good mathematics.

For the application of statistical methods the biologist finds an excellent review by H. L. Dunn (1929) with ten "don't's" and two "be sure of's." These twelve commandments ought to be instilled into every student of quantitative biology and physiology.

Benedict's table 4 on p. 175 (1938) contains an error which may lead to some confusion and is therefore mentioned here. The metabolic rates of the animals lighter than 1 kg. seem to have been calculated per unit of the various power functions of body weight: $W^{0.62}$, $W^{2/3}$, $W^{0.72}$ etc., on the basis of the erroneous assumption that generally (weight in g.)^p equals 100 (weight in kg.)^p. For $p = 2/3$ the result happens to come out all right because $1000^{2/3} = 100$ but for all the other powers that calculation is wrong; obviously it would be wrong for $p = 1$, since 1 kg. contains 1000, not 100 grams. The one kcal. of heat produced daily by the 8 gram dwarf mouse amounts to 39.2 kcal. per kg.$^{0.76}$ not 20.6 kcal. as listed in the table mentioned.

The great amount of material on metabolic rates, secured by reliable measurements in Benedict's laboratory, and condensed to a logarithmic chart on the relation of body size and metabolic rate (Benedict (1938) p. 171) is an excellent confirmation of Kleiber's earlier results (1932, p. 321). The two regression lines expressing the mean trends of these two sets of data are practically identical.

B. Check with recent data. To reinvestigate the relation of body size and metabolic rate among mammals, I have compiled 26 groups of metabolic rates measured under apparently comparable conditions. The animals were mature, in postabsorptive condition, measured in the range of metabolically indifferent environmental temperature, and at rest, or at least without abnormal activity. No data were used that were already incorporated in the earlier study (1932).

The basic data and the source of the material are given in table 2.

In figure 1 the logarithms of metabolic rate are plotted against the logarithms of body weight. The results used for calculating the regression line are indicated by dots in circles; those that are not used in the calculation are marked with brackets.

The reasons for not including these data in the calculation are as follows:

The result of only one shrew is so far reported and it is questionable whether the conditions of measurement allow a direct comparison with standard metabolic rate of the other animals. The Swiss mice were not in postabsorptive condition as indicated by a mean respiratory quotient of 0.96. Dwarf mice and growth hormone rats have an abnormal endocrine system. The result for swine was calculated from a mean net energy requirement for maintenance. The steer calves may be regarded as not yet mature and therefore their metabolic rate not strictly comparable with that of the other groups of animals. The conditions of measurement of metabolic rate of elephant, porpoise and whale are not strictly in line with the normal conditions of measuring standard metabolic rates.

Unfortunately, a lot of valuable data on man could not be incorporated in our chart because the results were given only per square meter of body surface. This is particularly true for the material of Boothby and Sandiford (1924), and the more recent findings of Young, Pittman, Donelson, and Kinsman (1943).

For the 26 comparable results, the method of least squares leads to the following linear regression equation:

$$\log M = 1.83 + 0.756 \log W \pm 0.05$$

where M = metabolic rate of animal in kilocalories per day

W = body weight in kilograms.

The regression coefficient of 0.756 ± 0.004 indicates that for the 26 results compiled in our table, the metabolic rate is most nearly proportional to the 0.756 or close to the 3/4 power of body weight.

The same interpolation of the earlier data (Kleiber, 1932) on ten groups of mammals leads to the equation:

$$\log M = 1.87 + 0.739 \log W \pm 0.03$$

TABLE 2

GROUP	ANIMAL	AUTHOR	BODY WT.	METABOL. RATE PER DAY
	a. Data used for calculation of regression line			
			kg.	*kcal*
1	Mouse	Benedict and Lee, 1936	0.021	3.6
2	Rat 230–300 days old	Kleiber, unpubl.	0.282	28.1
3	Guinea pig	Benedict, 1938	0.410	35.1
4	Rabbit	Tomme and Loria, 1936	2.98	167
5	Rabbit	R. Lee, 1939	1.52	83
6	Rabbit		2.46	119
7	Rabbit		3.57	164
8	Rabbit		4.33	191
9	Rabbit		5.33	233
10	Cat	Benedict, 1938	3.00	152
11	Macaque	Benedict, 1938	4.2	207
12	Dog	Galvão, 1942	6.6	288
13	Dog		14.1	534
14	Dog		24.8	875
15	Dog	de Beer and Hjort, 1938	23.6	872
16	Goat	Benedict, 1938	36.0	800
17	Chimpanzee	Bruhn and Benedict, 1936	38.0	1090
18	Sheep ♀	Lines and Peirce, 1931	46.4	1254
19	Sheep ♂		46.8	1330
20	Woman	McKittrick, 1936	57.2	1368
21	Woman	Lewis, Iliff and Duval, 1943	54.8	1224
22	Woman	McCrery, Wolf and Bavousett, 1940	57.9	1320
23	Cow	Benedict and Ritzman, 1935	300	4221
24	Cow	Kleiber, Regan and Mead, 1945	435	8166
25	Beef heifers	Kleiber, Goss and Guilbert, 1936	482	7754
26	Cow	Benedict and Ritzman, 1935	600	7877
	b. Data not used for calculation because conditions not comparable			
	Shrew	Morrison and Pearson, 1946	0.0035	2.9
	Swiss mice	U. S. Navy Res. Unit and Kleiber, 1944	0.0105	3.7
	Dwarf mouse	Benedict, 1938	0.008	1
	Rats (giant)	Benedict, 1938	0.400	33.2
	Rats (growth hormone)	Kleiber and Cole, 1939	0.391	28.6
	Swine	Breirem, 1936	150	2678
	Steer calves	Mitchell et al., 1940	200	3817
	Elephant	Benedict, 1938	3672	49000
	Porpoise	Irving et al., 1941	170	6768
	Whale	Irving, 1941	70000	1.2×10^6

The two bands, described by the two regression equations with their standard error of estimate, could be distinguished only by extrapolation to animals weighing less than 4 grams or more than 800 tons.

The figure shows the line by which the results would have to be represented if the metabolic rate were proportional to body weight, and also the line which would summarize the results if the metabolic rate were proportional to the 2/3 power of body weight (or approximately body surface).

The line expressing proportionality of metabolic rate to body weight stays within the band expressing metabolic rates proportional to the 3/4 power of

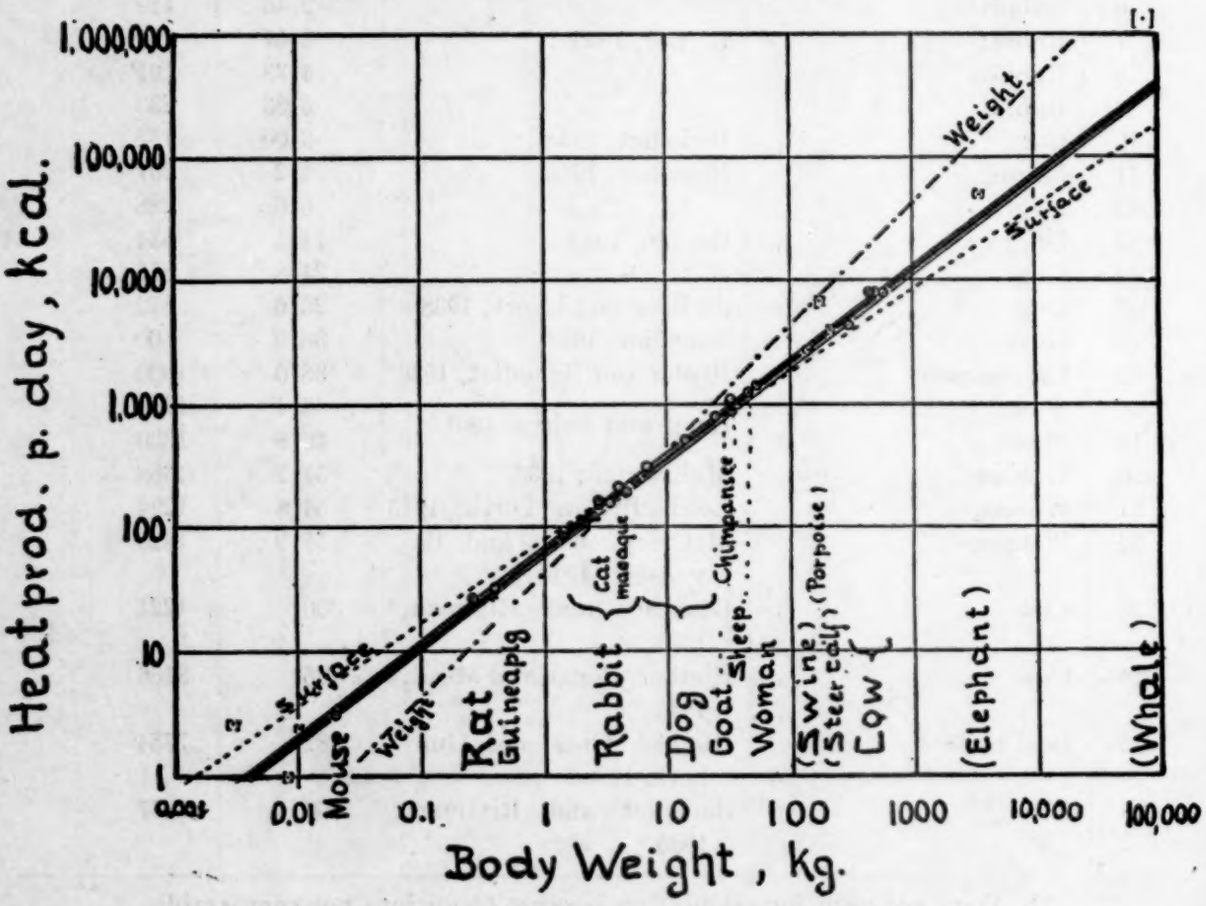

Fig. 1. Log. metabol. rate/log body weight

weight over a ratio of weights of 3.2. That means one would not be able to decide whether metabolic rates are proportional to body weight or proportional to its 3/4 power, unless the heaviest animals studied would weigh at least three times as much as the lightest animals.

Similarly, one may calculate that a significant difference between proportionality to the 3/4 power of body weight and proportionality to the 2/3 power of body weight (representing roughly the surface area), could not be established with groups of animals in which the heaviest animals weighed less than 9 times as much as the lightest animals.

C. Intraspecific comparisons. Since the differences in size have to be so con-

siderable to allow a significant decision of the question, whether the metabolic rate is more nearly proportional to the 2/3 or the 3/4 power of body weight, only a few species show great enough differences in mature weight for the establishment of the best fitting power function of body weight as basis for metabolic body size. This is true, even though the variability of metabolic rate for a given size is less within a species than between species.

In three species, namely, mice, rabbits, and dogs, the differences in size are considerable, and an analysis of the relation between body size and metabolic rate within these 3 species seems more promising than in the other species with more uniform size.

The regression line given for metabolic rate of mice (Benedict, 1938, p. 65) as a function of their body weight, would indicate a best fitting power function of body weight for metabolic body size

$$p = \frac{\Delta \log \text{metabol. rate}}{\Delta \log \text{body weight}} = 0.89$$

Benedict's line seems, however, definitely affected by a group of 4 endocrinologically abnormal dwarf mice. If we estimate the line through the other results, the best fitting power function would be 0.76.

The regression line given for rats (l.c. p. 67) would indicate that the rats intraspecifically followed the surface law. Their metabolic rate would be most nearly proportional to the 0.67 power of body weight.

The results of the 5 groups of rabbits reported by Lee (1939) may be represented by the following regression line:

$$\log M = 1.763 + 0.82 \log W \pm 0.09.$$

This indicates that in this population, metabolic rate is most nearly proportional to the 0.82 power of body weight; the range of body size in these rabbits would, however, have to be about 4 times as great as it is, to demonstrate a significant departure of rabbits from the hypothesis that metabolic rate is proportional to the 2/3 power of body weight. This range would have to be considerably more than four times as large to demonstrate that the intraspecific relation between metabolic rate and body size in these rabbits contradicts the hypothesis that the metabolic rate is proportional to the 3/4 power of body weight.

The data on the 3 groups of dogs reported by Galvão (1942) indicate proportionality of metabolic rate to the 0.84 power of body weight, and since the variability in this case seems rather small, the deviation from the surface law and even from the 3/4 power rule appears significant; so does the deviation from the hypothesis that the metabolic rate of these dogs is proportional to their body weight.

The result of Galvão contradicts the one which can be calculated on the basis of the material of Lusk, Kunde, and Steinhaus (see Benedict 1938 p. 79). Selecting from the regression line of this material, given by Benedict, the points for 9 kg. dogs and that for 18 kg. dogs, one may conclude that the metabolic rate of these dogs is most nearly proportional to the 0.55 power of body weight.

It is of interest to note that within the two groups of animals in which an intraspecific investigation of body size and metabolic rate appears most promising, rabbits and dogs, the metabolic rate seems to be most closely related to a power function of body weight higher than the 3/4. This problem deserves more study. Until there is a great deal more evidence for a difference in the intraspecific and the interspecific relation of body size and metabolic rate, the most rational temporary hypothesis is the one which is the simplest. That is the hypothesis that the intra- and the interspecific relations of body size and metabolic rate are the same. The question is whether the relation found intraspecifically should be assumed to hold also interspecifically, or vice versa. The interspecific derivation of the best suitable unit of metabolic body size seems, at present, the more reliable, because the great difference in size available by interspecific comparison makes size so dominant over other factors.

At present it appears, therefore, reasonable to adopt for intraspecific prediction of the metabolic rate the metabolic unit of body size found most suitable for interspecific prediction, namely, the 3/4 power of body weight.

METABOLIC BODY SIZE AND PREDICTION OF METABOLIC RATE. The metabolic body size is that function of size of animals to which the metabolic rate is proportional. Once the unit of metabolic body size is established, then the metabolic rate can be predicted by multiplying the metabolic body size with a given factor.

According to the surface law, the metabolic rate is proportional to the surface area. The metabolic body size, consequently, would be expressed in square meters of body surface, and according to Rubner's rule, the daily metabolic rate in kilocalories is 1000 times the number of square meters of body surface.

Since body surface is ill defined, the square meter of body surface is no suitable unit of size, even though it undoubtedly offers the great advantage of being easily visualized. Admittedly, it is easier to talk of heat production per square meter than to talk of heat production per unit of the 3/4 power of body weight in kilograms.

The Du Bois surface (1916) as calculated from weight and height is well defined within one species, but for comparison between man and rabbit, for example, one would have to rely again on the ill defined concept of "true" body surface.

Attempting to avoid hypotheses, such as the surface law, and ill defined terms, such as the body surface area, Harris and Benedict (1919) derived their empirical prediction equations, namely, (p. 227):

for men $h = +66.4730 + 13.7516w + 5.0033s - 6.7550a$

for women $h = +655.0955 + 9.5634w + 1.8496s - 4.6756a$

h = total heat production in kcal. per day, w = weight in kilograms

s = stature in centimeters and a = age in years.

Krogh (see Boothby and Sandiford, 1924, p. 80) has already criticised these equations.

That they allow an accurate prediction of the metabolic rate of men and

women similar to the material studied by Harris and Benedict is not in doubt, but physiologically the equations are practically meaningless.

Implied in these equations are several rather questionable assumptions; namely, that the metabolic rate of a weightless woman is 10 times that of a weightless man.

Further, it is assumed that metabolic rate is the sum of a linear function of body weight, a linear function of height, and a linear function of age.

Even though the Harris-Benedict equation deals with well defined quantities, such as weight, height, and age, Du Bois' prediction, despite its connection with the ill defined concept of body surface, seems to be more acceptable to clinicians as well as physiologists because it has a rational physiological meaning. Assuming that the metabolic rate is proportional to the 3/4 power of body weight, Kleiber (1932) re-analyzed the data of Harris and Benedict and developed the following prediction equations:

for men: $M = 71.2 \times W^{3/4} (1 + 0.004\ (30\text{-}A) + 0.010\ (S\text{-}43.4))$

for women: $M = 65.8 \times W^{3/4} (1 + 0.004\ (30\text{-}A) + 0.018\ (S\text{-}42.1))$

M = the metabolic rate in kilocalories per day

W = the body weight in kilograms

A = the age in years

S = the specific stature in $\dfrac{\text{centimeters height}}{W^{1/3}}$.

The equations have the following meaning: the metabolic rate of a man 30 years of age and with a specific stature of 43.4 cm./kg.$^{1/3}$ has a metabolic rate 71.2 times his body weight in kilograms raised to the 3/4 power. A woman of a standard age of 30 and standard specific stature for women (which is 42.1 or slightly less than that for men) has a metabolic rate of 65.8 per kg.$^{3/4}$. The ratio of 71.2 to 65.8 for the metabolic rates per unit of the 3/4 power of body weight of standard man and standard woman indicates the effect of sex on human metabolic rate. The second term in the parenthesis indicates that in adult man the metabolic rate decreases about 0.4 per cent of the metabolic rate of standard man for each year above (or increases for each year below) the standard age of 30.

It might have been preferable to express this age effect in relative terms, such as $0.12\dfrac{30\text{-}A}{30}$. Then the metabolic effect of age, expressed by the coefficient 0.12, would be more comparable with that in other animals with different standard age. (The relation of relative ages in different animals has been extensively studied by Brody (1945, chapter 19). Human metabolic rate would change 12 per cent per unit of relative departure from standard age (that is per 30 years). The last term in the parenthesis indicates that the mean specific stature (in the material of Harris and Benedict) for men is 43.4 cm. per kg.$^{1/3}$ that for women 42.1 cm.per kg.$^{1/3}$. Each centimeter per kg.$^{1/3}$ increase in specific stature produces, on the average, an increase of 1 per cent of the metabolic rate of men, and of 1.8 per cent of the metabolic rate of women. This suggests that the degree of slender-

ness (specific stature) affects the metabolic rate of women more than it affects the metabolic rate of men. The equations of Kleiber predict the metabolic rate of human beings with about the same accuracy as the empirical multiple regression equations of Harris and Benedict. The major advantage of the newer equations is that all terms have a physiological meaning.

Kleiber (1932) was somewhat vague about choosing a definite power function of body weight as the best basis for metabolic body size, and stated, p. 336, "that further investigation may show that some unit other than $W^{3/4}$ may be preferable."

Brody and Procter (1932) ventured a more definite formulation, namely:

$$M = 70.4\ W^{0.734}$$

and Brody, Procter and Ashworth (1934) obtaining exactly the same equation with a wider range of animals, including elephants, were confident that this "equation approaches closely the true relation between basal metabolism and body weight of mature mammals."

This relation was widely accepted (for example, in Höber's book, *The physical chemistry of cells and tissues*, p. 375). The National Research Council conference on energy metabolism (1935) endorsed the power function, after reducing it to two decimals (0.73), as the most suitable unit of metabolic body size. There was some doubt as to whether or not the data available justified the change from the 3/4 to the more complicated 0.73 power, a change which implied that the second decimal of the exponent was significant.

Taking into account a coefficient of variation in metabolic rate per unit size of 7 per cent, one may calculate that there is no significant difference between Brody's prediction and $72W^{3/4}$ within a group of animals ranging from a 10 gram mouse to a 16 ton super elephant. Within this range, there is thus not much point in discussing the question, whether the 0.734 power or the 3/4 power of body weight fits the metabolic results more closely.[4]

Recently, Brody (1945, p. 373) dropped also the second decimal from the exponent and suggests that the 0.7 power be used as reference for basal metabolism measurements.

Our recent analysis favors again the 3/4 power of body weight as the metabolic unit of body size. Aside from its empirical justification, the 3/4 power is preferable to either of Brody's successive 0.734, 0.73 or 0.7 because it is mathematically simpler since it can be calculated without logarithms.[5]

The daily fasting heat production per kg.$^{3/4}$ was:

in 10 groups of mammals, 1932: 71 ± 1.8 kcal. per kg.$^{3/4}$
in 26 groups of mammals, 1947: 69 ± 1.5 kcal. per kg.$^{3/4}$
together:
36 groups of mammals, 69 ± 1.2 kcal. per kg.$^{3/4}$

[4] The classification of the 3/4 power of body weight as the "Brody-Kleiber unit" (Günther, 1944) nevertheless is confusing. If these units have to be named according to authors at all, then Brody's unit would be the 0.734, 0.73 or 0.7 power, and Kleiber's the 3/4 power.

[5] $W^{3/4}$ can be easily obtained on a slide rule (extract the square root of the square root of the cube of the body weight).

For all practical purposes, one may assume that the mean standard metabolic rate of mammals amounts to 70 times the 3/4 power of their body weight (in kg.) per day, or about 3 times the 3/4 power of their body weight (in kg.) per hour.

BODY SIZE, FOOD REQUIREMENT AND DOSAGE OF BIOTICS. Two animals may be regarded as being on the same level of food intake when their rate of intake of metabolizable food energy[6] is the same multiple, or the same fraction, of their standard metabolic rate. Since the standard metabolic rate is proportional to the metabolic body size, or the 3/4 power of body weight, two animals may also be regarded as being on the same level of food intake, when they consume the same amount of metabolizable food energy per $kg.^{3/4}$

Not only the requirement of food energy, but also that of protein and of most vitamins, may be expressed per unit of the 3/4 power of body weight, because these dietary requirements are directly related to energy metabolism (see review by Kleiber 1945–46, p. 207).

For the dosage of drugs one should know whether or not the action depends on reaching a certain concentration in the blood stream without regard to its further maintenance. In this case the dosage should be proportional to body weight, since the amount of blood is proportional to body weight. If, however, the action of the biotic depends on the maintenance of a given concentration over a period of time, and if the rate of destruction or excretion of the biotic is proportional to the metabolic rate, then the dosage should be based on the metabolic body size.

The treatment may be so arranged that doses proportional to body weight are given and that the frequency of application depends on body size, so that over a sufficient period of time the rate of intake is proportional to the metabolic body size (the 3/4 power of body weight). In this case the frequency should be inversely proportional to the fourth root of body weight.[7]

If, for example, a 50 gram rat received one unit of a biotic daily, then to establish similar conditions under the assumptions made, a 500 kg. steer should receive a dose of 10,000 units every ten days.

BODY SIZE AND FOOD UTILIZATION. The establishment of a metabolic unit of body size is particularly advantageous for the investigation of food utilization.

$$\text{The quotient: } \frac{\text{energy in animal product}}{\text{total food energy}} = \frac{G}{U}$$

is called the total efficiency of food utilization. It measures (as far as that is possible in terms of energy) the success of animal husbandry.

[6] Metabolizable energy = heat of combustion of food minus heat of combustion of feces, urine, and methane.

[7] If q = amount given at one time (dosage)
f = frequency of application
W = body weight
then rate of intake = $f \cdot q = k_1 \cdot W^{3/4}$; $q = k_2 \cdot W$

$$f = \frac{k_1}{k_2} \cdot \frac{W^{3/4}}{W} = \text{const.} \cdot W^{-1/4}$$

The quotient $\frac{\Delta G}{\Delta U}$ is the partial efficiency. It is in general dependent on the level of food intake, but the discussion in this chapter may be limited to the simplest special case, that in which the partial efficiency is constant $= e$.

An animal needs a certain amount of food energy, U_m, to maintain itself without gain or loss of body substance. Only that amount of food energy consumed in excess of this maintenance requirement is available for production. The energy in the animal's production is, thus, the partial efficiency times the food energy available for production:

$$G = e(U - U_m)$$

therefore the total efficiency is:

$$\frac{G}{U} = e\left(1 - \frac{U_m}{U}\right)$$

The maintenance requirement U_m is related to the rate of fasting metabolism, B. The amount, B, of energy in body substance which the fasting animal would lose, is saved from being lost by the food energy U_m. The quotient $\frac{B}{U_m}$ is therefore the partial efficiency of food utilization for maintenance. Assuming for simplicity that this partial efficiency is the same as that for production, then $\frac{B}{U_m} = e$ or $U_m = \frac{B}{e}$.

Introducing this expression in the equation for total efficiency one obtains

$$\frac{G}{U} = e\left(1 - \frac{1B}{eU}\right), \quad \text{or } \frac{G}{U} = e - \frac{B}{U}$$

That means the total efficiency of food utilization is the difference between the partial efficiency (characterizing the nutritive content of the food), and the ratio between the basal metabolic rate and rate of intake of food energy. This ratio characterizes the capacity of the animal to take in food. Partial efficiency of utilization of metabolizable food energy (U) is related directly to the calorigenic (or specific dynamic) effect of food, ΔQ:

$$e = \frac{\Delta G}{\Delta U} = 1 - \frac{\Delta Q}{\Delta U}$$

There is no reason to assume that partial efficiency depends on body size. To the contrary, there is a good deal of evidence against such an assumption. Chambers and Lusk (1930) and Eaton, Cordill, and Gouaux (1936) for example observed that the specific dynamic action of glycine, administered to dogs varying from 5 to 13 kg., was independent of body size. The ratio $\frac{U}{B}$ has been defined as the "relative food level," and if an animal eats to capacity then $\frac{U}{B}$ may be termed the "relative food capacity" in terms of the basal metabolic rate.

Since the basal metabolic rate is proportional to the 3/4 power of body weight ($W^{3/4}$) the term $\frac{U}{W^{3/4}}$ characterizes the relative food capacity of the animal in terms of metabolic body size. Aside from differences in partial efficiency that animal is the better food utilizer which has the greater relative food capacity. The food intake per kg.$^{3/4}$, and similarly the gain per kg.$^{3/4}$, should be important characteristics for selection in breeding farm animals.

Since there is no reason to assume that partial efficiency depends on body size, the question of food utilization and body size is mainly a question of whether or not relative food capacity depends on body size. The simplest hypothesis is that the relative food capacity is independent of body size. Kleiber (1933), based on data obtained by himself and others, produced some empirical evidence in support of this simple hypothesis. By extensive experiments, Brody and his co-workers have confirmed this hypothesis for lactation (Brody and Proctor, 1935; Brody and Cunningham, 1936). Brody and Nesbit (1938) reported that energetic efficiency of rats (for lactation) is within that of cows. The efficiency for mechanical work is the same for large and small horses (Brody and Cunningham 1936) and for large and small men (Robinson, 1942).

A corollary to the hypothesis that total efficiency of food utilization is independent of body size, is the postulate that the relative rate of animal production is independent of body size. The relative rate of animal production may be the rate of gain in body substance per kg.$^{3/4}$, the rate of mechanical work per kg.$^{3/4}$ or the rate of milk production per kg.$^{3/4}$ (Kleiber and Mead, 1941, 1945).

Kleiber's hypothesis does not, of course, exclude the possibility that there are good and bad food utilizers. The meaning of the hypothesis is that the comparisons of good and large food utilizers with small and bad food utilizers, or vice versa, should not be used to establish a relation of body size and food utilization.

Similarly, one cannot directly compare young small animals with old large animals, even though in some cases age may not affect the relative production level. This is true, for example, for a considerable range in pigs. From figures given by Breirem (1939), one may calculate that the relative rate of gain for a 20 kg. pig is 112 kcal. per kg.$^{3/4}$; that of a 100 kg. pig, 118 kcal. per kg.$^{3/4}$.

SUMMARY AND CONCLUSIONS

1. Among homeotherms, from mice to cattle, metabolic rate and body size are correlated. This correlation is especially high when the metabolic rates are measured under standard conditions.

2. The metabolic rate of large and small homeotherms is more nearly proportional to the area of their respective body surfaces than to their body weights. This relationship is known as the surface law.

3. From five types of theories interpreting the surface law three are rejected, namely:

a. The theory based on the erroneous idea that the summated area of internal surfaces, such as the surfaces of the cells and the pulmonary alveoli, is proportional to the surface area of the body. The similarity in build of large and small animals cannot be extended to the dimensions of cells or alveoli.

b. Rejected also is the theory which bases the surface law on alleged differences in the chemical composition of large and small animals, involving such vague concepts as the "active protoplasmic mass". Neither does the theory which makes metabolic rate a function of anatomical composition stand the test. Uncritical evaluation of empirical data has led to the ill conceived generalization that the weights of the blood, the major glands and other organs are proportional to the 2/3 power of body weight or to the surface area. Logical application of this generalization can lead to absurd results.

c. The theory relating the surface law to an allegedly genetically fixed constant oxygen requirement of the cells, has rather little value for a physiological interpretation of the surface law. This theory, moreover, is in opposition to the well known fact that the metabolic rate of animals is essentially affected by somatic conditions. Furthermore, the metabolic rates of genetically closely related homeotherms of different size differ more than do the metabolic rates of genetically very different homeotherms but with the same body size. Admittedly, the concepts of "genetically similar or different" may change in the future when the biochemistry of the gene is further developed.

4. Two of the five types of theories on surface law are basically sound, namely, the theory connecting metabolic rate with rate of heat transfer and the theory relating metabolic rate to blood circulation. These theories may be integrated into one as follows: *In natural selection, those animals prove to be better fit whose rate of oxygen consumption is regulated so as to permit the more efficient temperature regulation as well as the more efficient transport of oxygen and nutrients.*

5. This theory does not postulate a strict proportionality between the area of a "true" body surface and metabolic rate. Body surface area has been used in very valuable work on animal heat exchange (Rubner, 1902; Deighton, 1933; Winslow et al., 1934–39; Burton, 1934; DuBois, 1937; Hardy and DuBois, 1938). As a basis for comparing metabolic rates of large and small animals, however, body surface area is not well enough defined. The use of an ever increasing variety of surface areas, even for the same species of animals, has led to an unnecessary and deplorable state of vagueness in comparative physiology of metabolism.

6. Relatively recent results on homeotherms, ranging from mice to cattle, indicate that the metabolic rate per unit of the surface area is greater the larger the animal. A linear correlation between the logarithm of metabolic rate and the logarithm of body weight shows that metabolic rate is proportional to a given power function of body weight. The metabolic rate divided by the 3/4 power of body weight is independent of body size.

7. The 3/4 power of body weight is therefore recommended as representative of metabolic body size, and "kg.$^{3/4}$" chosen as the symbol for the unit. The body weight in kilograms, raised to the 3/4 power, measures the metabolic body size of an animal in kg.$^{3/4}$.

8. The metabolic level of an animal may be characterized as the metabolic rate per kg.$^{3/4}$. Under standard conditions the metabolic level of adult homeotherms, from mice to cattle, averages 70 kcal. per kg.$^{3/4}$ per day or about 3 kcal per kg.$^{3/4}$ per hour.

9. At present there seems to be no sufficient reason against the intraspecific application of the 3/4 power rule of metabolic rate. Modulating factors for age and specific stature may be incorporated into prediction equations for human metabolic rate based on the 3/4 power rule. Such equations are preferable to the irrational regression formulas of Harris and Benedict, and they avoid the connection with the ill defined body surface involved in the Du Bois prediction.

10. The unit of metabolic body size is useful for expressing levels of food intake and of animal production, it is a sound basis for comparing food capacity and production capacity of animals that differ in body size. Relative food capacity (maximum rate of food intake per $kg.^{3/4}$) and relative production capacity (maximum rate of production per $kg.^{3/4}$) should be among the most important criteria for selecting efficient food utilizers. The metabolic body size may also be useful in estimating dosage of biotics.

11. When the concepts concerned with the relationship of body size and metabolic rate are clarified, and when not only the methods of measurement, but also those of reporting the data are sufficiently standardized, then comparative physiology of metabolism will be of great help in the efforts to solve one of the most interesting and intricate problems of biology, the regulation of the rate of cell metabolism.

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PHARMACOLOGY OF ANTIHISTAMINE COMPOUNDS

EARL R. LOEW

Department of Pharmacology and Experimental Therapeutics, University of Illinois College of Medicine, Chicago

Those drugs which are capable of diminishing or preventing several of the pharmacological effects of histamine and which do so by a mechanism other than the production of pharmacological responses diametrically opposed to those produced by histamine may be termed *histamine antagonists* or *antihistamine drugs*. Epinephrine and its congeners are not considered as antihistamine drugs, being excluded by definition since they induce prominent effects such as bronchiodilatation, vasoconstriction, decreased capillary permeability, inhibition of intestinal activity, etc., which represent the antithesis of those produced by histamine. True antihistamine drugs, on the other hand, are able to antagonize histamine without eliciting pharmacological responses, or if responses are elicited they do not appear to be of the type or degree which suggest an important causal relationship to the histamine antagonism. Excitatory sympathomimetic activity is not essential for antihistamine action since certain adrenergic blocking drugs are capable of antagonizing histamine. Use of the term "histaminolytic" with reference to drugs which antagonize histamine has been criticized (36) since there is no evidence that the drugs referred to are capable of destroying or neutralizing histamine.

In the present discussion, emphasis will be placed on those drugs which are potent histamine antagonists and which, from the evidence available, appear to act as blocking agents with some degree of specificity as regards antihistamine action. Pharmacological experience reveals that specificity is relative and not absolute, the latter being rare or practically unattainable. Atropine and other antispasmodic drugs, epinephrine and related amines, and certain amino acids and derivatives of histamine are capable of antagonizing some of the effects of histamine (for refs. cf. 78, 142, 150, 151). However, these compounds can be regarded as non-specific since their ability to contract or relax smooth muscle and to antagonize acetylcholine, barium and other spasmogenic agents equals or exceeds their effectiveness in opposing histamine.

The motivation for the development of antihistamine drugs undoubtedly came from the realization that histamine, omnipresent in tissues and able to produce diverse effects when minute quantities are liberated therefrom, possibly acts as a mediator or regulator of physiological processes and as a culprit in certain pathological states. Several reviews cite much of the literature dealing with the questions concerning the involvement of histamine in various physiological mechanisms (10, 43, 46, 166). Problems to be clarified include the rôle which histamine may play in mediating vasodilator nerve impulses (96), in altering capillary permeability (97, 102), in producing gastric secretion (51, 73, 87), pain (157), and reactive hyperemia (5).

Recent reviews discuss evidence related to the question as to whether histamine is one of the factors involved in producing certain manifestations in pathological conditions (10, 32, 43–46, 60, 149, 150, 166). Opinions concerning the rôle of histamine in various types of inflammatory reactions vary widely (13, 102, 131, 150, 152, 153, 159). There is an obvious need for determining the extent to which histamine may be involved in radiation effects (13, 114), certain types of headache (166, 188), gastric and duodenal ulcers, traumatic, burn and surgical shock (185), pruritus (25, 135, 159), responses to a variety of venoms (54, 94, 150), and toxemias of pregnancy. In the latter condition, and during normal pregnancy, there is the possibility that the metabolism of histamine and histidine may be appreciably altered (3, 92, 93). Prevalence of allergic diseases and the evidence which suggests that histamine accounts for some of the manifestations also emphasizes the importance of the development of antihistamine drugs.

In 1940 and 1941, the extensive critical reviews by Dragstedt (43, 44) and Feldberg (60) discussed the evidence which convincingly supported the hypothesis that histamine is liberated from tissues by the antigen-antibody reaction and accounts for the important symptoms of anaphylaxis. Likewise, in allergic diseases, a variety of alterations in tissues and fluids resemble the pharmacological responses to histamine and it is customary to consider the symptoms of allergy as resulting from an antigen-antibody reaction. Code (32), Rocha e Silva (149, 150) and Dragstedt (45) have recently presented additional evidence in support of the contention that histamine plays a prominent rôle in anaphylaxis and allergy.[1] These authors were cognizant of the fact that the evidence was indirect and circumstantial. All were aware of the need for additional evidence, even though indirect in type. The minute amounts of histamine which are capable of eliciting responses are probably not always detected by available chemical and biological methods.

Interest in antihistamine drugs is related to their use: *a*, as pharmacological tools for differentiating between effects of histamine and those due to other agents; *b*, as therapeutic agents to alleviate symptoms due to histamine, i.e., in allergy and possibly other conditions, and *c*, as diagnostic agents to aid in proving or disproving that symptomatology is referable to histamine or to other causes.

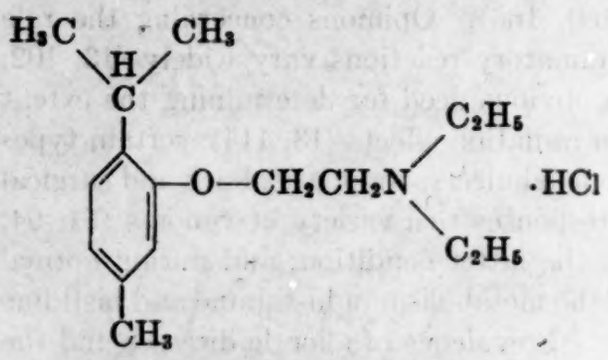
The use of antihistamine drugs in elucidating these numerous problems obviously necessitates an adequate knowledge of their pharmacological and toxicological properties, mode of action, degree of specificity, and in the absence of absolute specificity, a due consideration of other than antihistamine action.

The antihistamine drugs herein discussed will be identified before proceeding to a more detailed discussion of pharmacological action and toxicity.

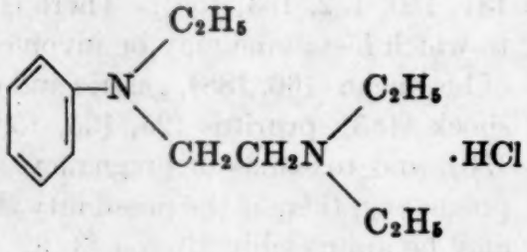
Fourneau compounds (929 F and 1571 F). 2-Isopropyl-5-methylphenoxyethyldiethylamine (929 F) is a phenolic ether which is frequently designated as thymoxyethyldiethylamine. Following the preliminary reports from Doctor

[1] The failure to detect an elevated blood level of histamine does not negate its involvement in tissue reactions since it leaves the blood stream with extreme rapidity (32,47,155) and extraction methods do not always detect physiologically active amounts (53, 115).

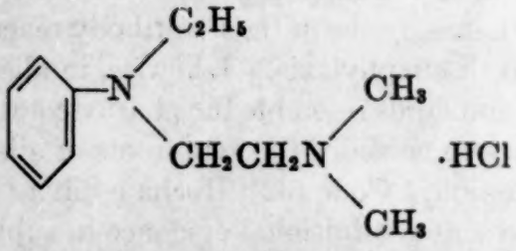
Bovet's laboratory[2] at the Pasteur Institute in Paris concerning the antihistamine and anti-anaphylactic properties of 929 F (23, 170), Anne-Marie Staub (169)



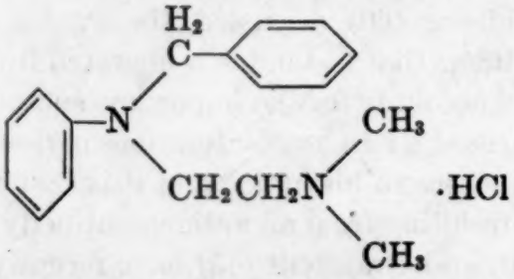
2-Isopropyl-5-methylphenoxy-ethyldiethylamine·HCl
(Fourneau 929)



N-Phenyl-N,N′,N′-triethylethyl-enediamine·HCl
(Fourneau 1571)



N-Phenyl-N-ethyl-N′,N′-dimethyl-ethylenediamine·HCl
(2325 Rhône-Poulenc)



N-Phenyl-N-benzyl-N′,N′-dimethyl-ethylenediamine·HCl
(2339 Rhône-Poulenc) Antergan

published extensive data relative to the antihistamine properties of several series of compounds synthesized by E. Fourneau. 929 F proved to be the most effective of the phenoxyethylamines with respect to alleviation of the symptoms of histamine shock in guinea pigs, but was slightly less effective than 1571 F, N-phenyl-N,N′,N′-triethylethylenediamine, selected from the series of N-phenylethylene diamines. The extensive pharmacological and toxicological studies conducted with the most promising antihistamine compounds, 929 F and 1571 F, deserve special commendation in view of the demonstration that agents could be synthesized which would antagonize histamine and also diminish or annul certain anaphylactic reactions. Furthermore, their findings were soon corroborated (24, 31, 77, 107, 110, 111, 112, 156) and rapidly extended (18, 27, 75, 76, 106, 132, 158, 178, 186, 187). The diverse pharmacological actions and toxic effects produced by 929 F and 1571 F have precluded their use in therapeutics and extensive use as research tools. The success of the French investigators motivated the search for superior antihistamine compounds.

Antergan (2339 R.P.) and 2325 R.P. Evidence of definite progress in the

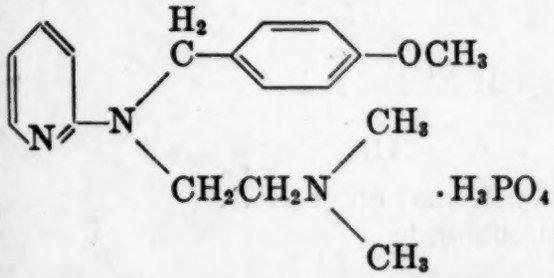
[2] The erroneous statement has been made (146) that the starting point for the development of antihistamine drugs began in 1933 with the report of Fourneau and Bovet (Arch. int. Pharm. et thérap., **46**: 178–191, 1933). However, the phenolic ethers considered were investigated for sympatholytic properties and no reference was made to their effects in antagonizing histamine.

development of more potent antihistamine compounds was presented by Halpern (77) who studied twenty-four derivatives of 1571 F and selected two compounds which exerted marked antihistamine action from the compounds synthesized by Mosnier in the laboratories of Rhône-Poulenc. The dimethyl homologue of 1571 F, N-phenyl-N-ethyl-N′,N′-dimethylethylenediamine (2325 R.P.), and N-phenyl-N-benzyl-N′,N′-dimethylethylenediamine (2339 R.P. or Antergan) were selected and studied extensively relative to antihistaminic, anti-anaphylactic, and antispasmodic action, as well as pharmacodynamic and toxicological properties. Of the aniline derivatives, 1571 F, 2325 R.P. and Antergan, the latter is the only compound which has been evaluated therapeutically in man (for ref. cf. 56, 146).

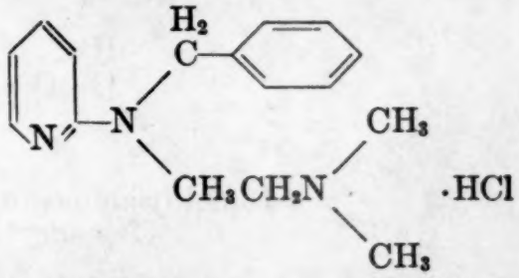
Antistine (2-N-phenyl-N-benzylaminomethylimidazoline), an analogue of Antergan, appears to possess pharmacological properties and exert therapeutic effects similar to those of Antergan (19, 25, 127, 162).

Since 1942, synthetic compounds other than aniline derivatives have been found to possess marked antihistamine properties and a sufficiently low toxicity as to permit effective use as research tools and therapeutic agents. The compounds which have been examined most extensively were selected from α-aminopyridine derivatives and benzhydryl alkamine ethers.

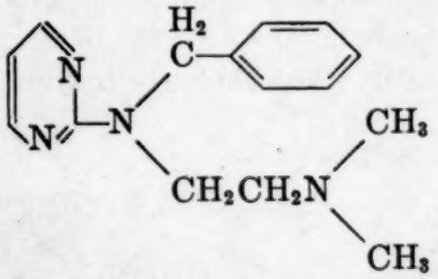
α-Aminopyridine derivatives. Definite progress was made by Horclois (cf. 24) who substituted a heterocyclic structure, pyridine, for the phenyl ring in Antergan. Bovet and co-workers (20, 21, 24), in 1944, found that N-α-pyridyl-N-p-



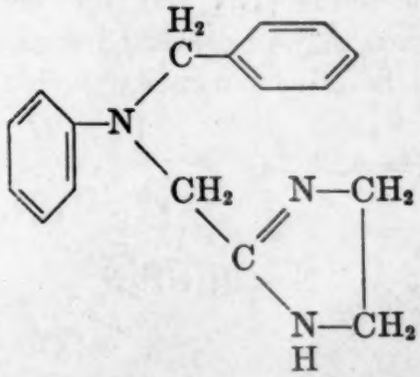
N-α-Pyridyl-N-p-methoxybenzyl-N′, N′-dimethylethylenediamine·H_3PO_4 (2786 R. P.) Neoantergan



N-α-Pyridyl-N-benzyl-N′,N′-dimethylethylenediamine·HCl Pyribenzamine hydrochloride



N-2-Pyrimidyl-N-benzyl-N′,N′-dimethylethylenediamine Hetramine



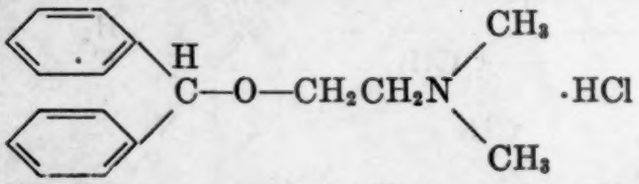
2-N-Phenyl-N-benzylaminomethylimidazoline Antistine

methoxybenzyl-N′,N′-dimethylethylenediamine (2786 R.P. or Neoantergan) possessed a remarkable degree of antihistamine potency, as shown by the relief or prevention of bronchospasm in guinea pigs induced by anaphylaxis or administration of histamine. This compound was found to be capable of diminishing or preventing most of the effects of histamine on smooth muscle and the vascular system.

In 1945, Mayer, Huttrer and Scholz (121, 122) reported the antihistamine and anti-anaphylactic action of a homologue of Neoantergan, N-α-pyridyl-N-benzyl-N′,N′-dimethylethylenediamine (63-C or Pyribenzamine hydrochloride). Pyribenzamine lacks the methoxy group on the benzyl ring contained in Neoantergan. The close chemical similarity between Neoantergan and Pyribenzamine would suggest similarities in the qualitative actions and toxicity of the two compounds which will be apparent from the discussion to follow.

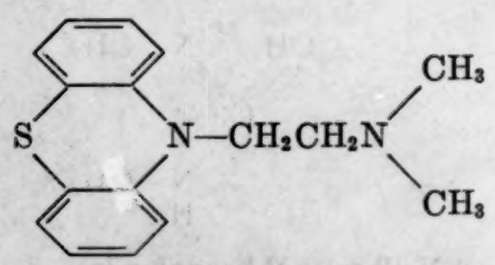
Hetramine, N-2-pyrimidyl-N-benzyl-N′,N′-dimethylethylenediamine, is a pyrimidine analogue of Pyribenzamine. In recent months, Feinstone, Williams and Rubin (59) demonstrated that Hetramine protected guinea pigs against histamine shock and anaphylactic shock.

Benzhydryl alkamine ethers. The availability of benzhydryl derivatives synthesized by Rieveschl and Huber (cf. ref. 110) which possessed an ether linkage and other structural characteristics similar to phenolic ethers such as 929 F, motivated the author, in 1943, to test the benzhydryl alkamine ethers for antihistamine action. β-Dimethylaminoethyl benzhydryl ether (Benadryl

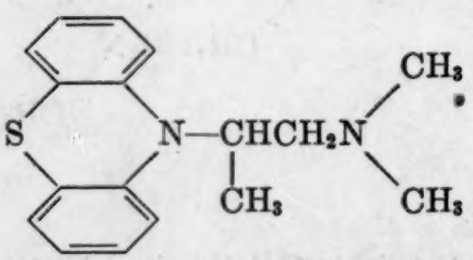


β-Dimethylaminoethyl benzhydryl ether·HCl
Benadryl hydrochloride

hydrochloride) and several related tertiary amines exerted definite antihistamine and anti-anaphylactic action (107, 110). Furthermore, it was demonstrated (109, 112, 187) that quaternary nitrogen derivatives of Benadryl were potent histamine antagonists and, although the quaternary compounds are choline derivatives, they also exerted an anti-acetylcholine or atropine-like action instead of the usual muscarinic action exerted by most choline ethers and esters (72.)



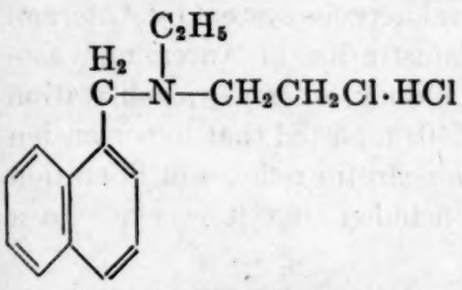
N-β-Dimethylaminoethyl-thiodiphenylamine
(3015 R. P.)



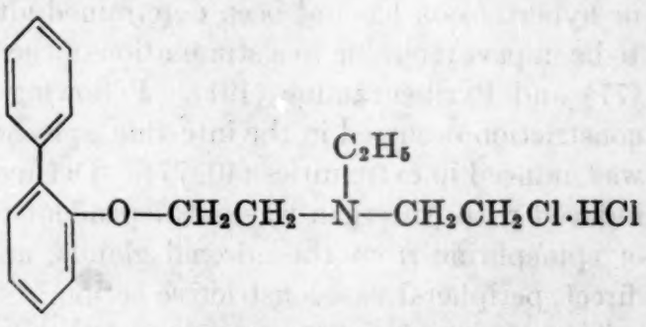
N-β-Dimethylaminopropyl thiodiphenylamine
(3277 R. P.)

Thiodiphenylamines. According to preliminary reports by Halpern and Ducrot (80) and Halpern (79), two derivatives of thiodiphenylamine (3015 R.P. and 3277 R.P.) protected guinea pigs, rabbits and dogs against exceedingly large doses of histamine injected intravenously. It was stated that these compounds antagonized the spasmogenic action of histamine on the uterus and intestine and the depressor action in the cat, dog, and rabbit. Salivary, pancreatic and gastric secretion (histamine-induced?) were not inhibited.

Other compounds. Of particular importance in relation to anti-anaphylactic effectiveness and mode of action of antihistamine drugs is the discovery (1) of a group of compounds which oppose several actions of histamine and, in contrast to all other potent antihistamine compounds which enhance the pressor action of epinephrine, these block and reverse the pressor action of epinephrine (adrenergic blocking action). α-Naphthylmethylethyl-β-chloroethylamine and β-2-biphenyloxyethyl-β-chloroethylethylamine[3] exert this dual action. They are effective in alleviating histamine shock and anaphylactic shock in guinea pigs and in diminishing the depressor responses to histamine in dogs. α-Naphthylmethylethyl-β-chloroethylamine and Neoantergan are equally effective (1) in preventing fatal histamine shock (M.E.D. of 0.025 mgm./kgm., subcutaneously) and anaphylactic shock in guinea pigs.



α-Naphthylmethylethyl-β-chloroethylamine·HCl



β-2-Biphenyloxyethyl-β-chloroethylethylamine·HCl

PHARMACODYNAMICS. Doses of antihistamine drugs commonly employed in animals do not exert a marked effect on respiration and the clinical literature contains no indication that doses recommended for therapeutic use in man have any effect (126, 146). An increased rate and amplitude of respiration persisted for some minutes following intravenous injection of 929 F, 1571 F (169) and Antergan (77) in dogs anesthetized with chloralose. Similar responses have been obtained with Neoantergan, Pyribenzamine and Benadryl (112, 167, 168, 191) in dogs anesthetized with pentobarbital or phenobarbital. A short period of apnea preceded the stimulatory phase when several of these drugs were injected rapidly (77, 112, 167). With all compounds excepting 929 F, it is doubtful that the respiratory alterations are reflex responses referable to changes in blood pressure since the brief apnea was usually concurrent with hypotension. Furthermore, respiratory stimulation persisted for some minutes

[3] Synthesized by Drs. G. Rieveschl, Jr., R. Fleming and W. R. Coleman of Parke, Davis and Company, Detroit, Michigan.

and, in general, was concurrent with a normal or slightly increased level of arterial blood pressure.

Following administration of large oral or parenteral doses of antihistamine drugs, respiratory stimulation has also been noted in unanesthetized animals (77, 105, 120). Such stimulation usually preceded or was concomitant with marked excitability, tremors and convulsions. Secondary respiratory depression may have been a contributory cause of death (120, 147). Mention was made of a general narcotic effect in rats which received large doses of Neoantergan (42).

The hypotension induced in anesthetized dogs, cats and rabbits with large doses of 929 F was frequently pronounced and of appreciable duration (31, 105, 169, 178), thus complicating interpretation of data relating to histamine antagonism. Hypertension was reported to occur in unanesthetized dogs (132) treated with 929 F (40 mgm./kgm., S.C.). More potent antihistamine drugs such as 1571 F, Antergan, Neoantergan, Benadryl and Pyribenzamine, induced a transient hypotension in dogs when injected intravenously at a rapid rate (77, 112, 167, 168, 169, 190). However, it is quite apparent that hypotension was minor in degree, absent, or replaced by a slight hypertension which persisted for several minutes when these drugs were administered slowly or by other routes (24, 77, 112, 137, 167, 169, 182, 191). The exact cause of the hypotension or hypertension has not been determined although hypertension was considered to be in part referable to a stimulation of the central nervous system by Antergan (77) and Pyribenzamine (191). Following administration of Antergan, vasoconstriction occurred in the intestine, spleen and kidney, whereas vasodilatation was induced in extremities (40, 77). DeCuyper (40) reported that hypertension induced with Antergan was not dependent on sino-carotid reflexes or liberation of epinephrine from the adrenal glands, and concluded that it was due to a direct, peripheral vasoconstrictive action.

The rise in arterial pressure in anesthetized dogs following intravenous injection of Antergan, Pyribenzamine, Neoantergan and Benadryl was slight in degree. Since the hypertension persisted for only a few minutes, it could scarcely be related to other actions of the drugs, such as antagonism of histamine, which was demonstrable for one or more hours. Doses of antihistamine drugs which are therapeutically effective in allergic diseases do not raise blood pressure (70, 126, 146). This indicates that antihistamine drugs lack the pressor properties possessed by epinephrine and its congeners.

The majority of antihistamine drugs exert some local anesthetic action (25, 31, 42, 77, 110, 157, 169), the degree of which has not been quantitatively determined except with Neoantergan (42). Local anesthetic action should be considered when interpreting effects of antihistamine drugs on vascular and cutaneous responses in which axon and other reflexes are involved. Such action could possibly be related to anti-pruritic effects and production of cutaneous analgesia (31, 42, 157, 158), the latter having been demonstrated following large doses of 929 F and Neoantergan in animals.

Very few studies have been made which concern the direct and indirect action of antihistamine drugs upon the heart. Neoantergan was reported to exert a

quinidine-like action on isolated auricular tissue from rabbits (42). In normal human subjects, oral administration of maximal therapeutic doses of Benadryl for several weeks seldom lowered systolic blood pressure and did not produce clinically detectable alterations of the heart (126).

Toxic doses of all antihistamine drugs, with the exception of quaternary derivatives of Benadryl (187), stimulate the central nervous system of animals as evidenced by hyperexcitability, tremors and convulsions (cf. toxic effects). It has been stated (29) that Antergan enhances the excitability of cortical fibers and peripheral motor and sensory fibers; these effects are in accord with the generalized excitation produced in animals by Antergan (77). In animals, antihistamine drugs do not induce sedation, hypnosis or depressant effects. In contrast to the hyperexcitability and convulsions induced in several species of animals with large doses of antihistamine drugs is the sedation and dizziness which sometimes obtain in human beings following administration of therapeutic doses. In man, sedation is unpredictable, variable in degree from person to person, and constitutes a side-effect which has been encountered frequently with Benadryl (9, 55, 58, 101, 123, 135, 146, 172), less frequently with Pyribenzamine (6, 7, 58, 63, 146) and which is known to occur following use of Antergan (37, 39) and Neoantergan (37, 38).

Untoward reactions in man which result from direct or indirect actions of antihistamine drugs on the gastrointestinal tract include gastric distress, nausea, emesis, colic and diarrhea. Such reactions are seldom encountered with Benadryl (9, 55, 101, 123, 135, 172) which exerts a slight degree of antispasmodic action (vide infra). They possibly occur more frequently with Antergan (28, 37, 163, 164), Neoantergan (37) and Pyribenzamine (6, 7, 58, 63, 146), i.e., with those drugs which are practically devoid of antispasmodic properties, but which are capable of inducing spasm of intestinal and uterine muscle of animals *in vitro* and *in vivo* (vide infra).

Antihistamine drugs are absorbed rapidly following oral and parenteral injection. The usual experimental and therapeutic doses exert antihistamine and anti-allergic action in animals and man for two to six hours. No information is available concerning effective blood levels and rate or mode of destruction or elimination. Analyses made by Gelvin and McGavack (71) revealed the presence of comparable quantities of Benadryl in the blood and spinal fluid of seven patients.

ACUTE AND CHRONIC TOXICITY. Those antihistamine drugs which are now being used as therapeutic agents were developed concurrently, or in rapid succession, which fact accounts for the minimal number of direct comparative studies relating to pharmacodynamics and toxicity. Comparisons of toxicity data obtained in different laboratories under varying conditions relative to technique and animals employed, may be misleading, especially when no reference compound is included in the studies which would at least permit a comparison of relative toxicity values. A direct comparison (167) of the acute toxicity following intraperitoneal injection of Neoantergan, Pyribenzamine and Benadryl in mice revealed no important difference in the values for LD 50 which ranged

from 75 to 90 mgm./kgm. Acute toxicity data (LD 50) reported by various investigators are included in table 1.

The tabulated data reveal that none of the antihistamine drugs are extremely toxic and it is unlikely that any major differences exist in relation to the acute toxicity of the various compounds. Furthermore, the ratio of these lethal doses to the small doses required to demonstrate antihistamine and anti-anaphylactic activity in animals consistently yields a high therapeutic index. The magnitude of the toxic doses of 929 F, 1571 F (31, 110, 169), and 2325 R.P. (24, 77) in

TABLE 1

Acute toxicity of antihistamine drugs (LD 50 in mgm./kgm.)

ANIMAL AND ROUTE OF INJECTION	ANTERGAN		NEOANTERGAN		BENADRYL		PYRIBENZAMINE	
	LD 50	(Ref. no.)	LD 50	(Ref. no.)	LD 50	(Ref. no.)	LD 50	(Ref. no.)
Mouse:								
i.p.			90	(167)	75	(167)	80	(167)
s.c.	175	(24)	150	(24)	130	(147)	75	(120)
oral					167	(147)	210	(120)
i.v.	40	(24)	30	(24)			12	(120)
Rat:								
i.p.					82	(110)		
s.c.	175	(77)					340 ♂ 225 ♀	(120)
oral	300	(77)			545	(147)	570 ♂ 515 ♀	(120)
i.v.					46	(147)	12	(120)
Guinea pig:								
s.c.	110	(24)	70	(24)				
i.p.					75	(110)		
Rabbit:								
s.c.							33	(120)
i.v.					10.5	(147)	9	(120)
Dog.:								
i.v.					30	(147)		

several animal species at least suggests that these drugs are no more acutely toxic in animals than compounds included in table 1, but 929 F produced marked side actions in dog and man (31, 169).

Toxic doses of antihistamine drugs in several animal species induced tremors, then convulsions which were sometimes followed by depression, and finally death which appeared to be due to cardio-respiratory depression. The exact cause of death should be determined, as well as means of antidoting toxic amounts of these drugs. The acute toxic effects of antihistamine drugs appear unrelated to any physiological mechanism involving histamine.

No indications of chronic toxicity were observed in studies on blood, urine, parenchymatous organs, nervous system or bone marrow of dogs receiving Benadryl orally (147) for several weeks in daily doses (40 to 60 mgm./kgm.) capable of causing nervousness, ataxia, gastro-intestinal reactions and hyperesthesia of the skin. In man, administration of Benadryl for as long as eighteen weeks did not cause variations in cells and other constituents of blood (126). Pyribenzamine has been administered orally to three human subjects for 80 days (95), to four dogs for one year and to rats for many months (117, 120) without inducing biochemical or pathological changes in body fluids, urine, or tissues. Doses of Neoantergan equivalent to the maximum human therapeutic dose, on the basis of body weight, were given to young rats in two injections daily for eleven days without affecting growth (42).

It is encouraging to note that the clinical literature referable to Antergan, Benadryl, Pyribenzamine, Neoantergan and Antistine yields no evidence as yet that they can induce blood dyscrasias or organic changes in the nervous system or parenchymatous organs.

ANTAGONISM OF HISTAMINE. A prominent pharmacological action of histamine is its spasmogenic action on smooth muscle of bronchioles, gastro-intestinal tract, uterus, ureters and gall bladder (10, 61, 72). Histamine induces arteriolar constriction or dilatation depending on animal species or locality of vascular bed in a given species. A conspicuous action of histamine is the production of increased capillary dilatation and greater capillary permeability, the latter factor being most important in the production of localized edema (97). Finally, histamine is a potent secretagogue which induces secretion from lacrimal, salivary, gastric and pancreatic glands. This secretagogue action is probably most important with reference to gastric secretion.

A consideration of antihistamine drugs must necessarily concern their ability to antagonize the various actions of histamine.

1. *Bronchioles.* Usually, antihistamine drugs have been selected and partially evaluated by determining their ability to alleviate bronchioconstriction which is so conspicuous in guinea pigs when histamine is injected or liberated during anaphylaxis (cf. 183). Until recently, it was difficult to compare the relative effectiveness of various drugs since various investigators used different techniques and frequently failed to include a standard or reference compound which would at least permit indirect comparisons of data from various sources. More recently, a majority of the antihistamine drugs were tested (65, 108, 110, 111, 167, 187) under conditions which were sufficiently standardized as to yield a remarkable consistency in the incidence of asphyxial deaths referable to histamine-induced bronchioconstriction in untreated guinea pigs, and which permitted a fairly reliable determination of the minimal effective dose (M.E.D.) of a given drug which was capable of partially annulling the action of histamine. Essentially, the method (110), a modification of that used by Kallós and Pagel (91) and others (77, 161), consists of determining the M.E.D. of a drug which significantly reduced the mortality rate of guinea pigs exposed to a lethal dose of an atomized histamine solution (see table 2).

The ability of 929 F and 1571 F to diminish the bronchioconstrictive effects of histamine in guinea pigs (23, 169, 170) has been confirmed by several investigators (31, 77, 110, 111, 156, 186). Antergan (77, 78) and 2325 R.P. were more effective. Antergan exerted a high degree of antihistamine specificity on the bronchiolar muscle of guinea pigs since even large doses failed to relieve bronchioconstriction induced with an aerosol of acetylcholine and eserine (77).

TABLE 2

Minimum effective doses of antihistamine drugs which reduce the incidence of mortality in guinea pigs exposed to an aerosol of histamine

DRUG	MOL. WT.	DOSE *	ROUTE	REF. NO.
		mgm./kgm.		
2-Isopropyl-5-methylphenoxyethyldiethylamine hydrochloride, 929 F (Fourneau)	285.80	6.0	i.p.	(110, 111)
		6.0	s.c.	(24)
N-Phenyl-N,N′,N′-triethylethylenediamine hydrochloride, 1571 F (Fourneau)	256.81	3.0	i.p.	(110, 111)
		5.0	s.c.	(77)
N-Phenyl-N-ethyl-N′,N′-dimethylethylenediamine hydrochloride, 2325 R.P. (Rhône-Poulenc)	228.75	2.5	s.c.	(77)
N-Phenyl-N-benzyl-N′,N′-dimethylethylenediamine hydrochloride, 2339 R.P., Antergan	290.81	1.0	s.c.	(24)
		0.5	s.c.	(77)
N-Pyridyl-N-p-methoxybenzyl-N′,N′-dimethylethylenediamine·H_3PO_4, 2786 R.P., Neoantergan	382.37	0.075	i.p.	(167)
		0.025	s.c.	(1)
		0.1	s.c.	(24)
N-α-Pyridyl-N-benzyl-N′,N′-dimethylethylenediamine hydrochloride, Pyribenzamine	291.79	0.3	i.p.	(167)
β-Dimethylaminoethyl benzhydryl ether hydrochloride, Benadryl	291.45	1.5	i.p.	(110, 111,) (112, 167)
β-Benzhydryloxyethyltrimethylammonium iodide	397.30	0.5	i.p.	(112, 187)
N-2-Pyrimidyl-N-benzyl-N′,N′-dimethylethylenediamine, Hetramine	255.33	0.3 to 0.6	i.p.	(59)
α-Naphthylmethylethyl-β-chloroethylamine hydrochloride	284.19	0.025	s.c.	(1)

* Doses employed by Halpern (ref. 77) were capable of preventing development of serious symptoms, whereas those employed by other workers diminished mortality. Pretreatment with drugs was instituted 15 to 30 minutes before exposure to histamine aerosol.

Large doses of epinephrine were required to oppose effects of either acetylcholine or histamine.

In rapid succession, it was demonstrated that Neoantergan (21, 24), Benadryl and related compounds (109–112, 167), Pyribenzamine (118, 122), Antistine (127) and Hetramine (59) were highly effective in antagonizing the bronchioconstriction induced in intact guinea pigs by exposure to a histamine aerosol. Most of these findings have been confirmed (65, 66, 105, 167), and each of these drugs was capable of antagonizing the bronchioconstriction induced by intravenous injections of histamine.

The relative potency of several antihistamine drugs has also been ascertained by determining the number of intravenously injected LD 50 doses of histamine which a fixed dose of drug will antagonize sufficiently to prevent asphyxial death in guinea pigs (24, 66, 80). In general, the same relative potency of the various compounds is obtained by administering histamine intravenously or as an aerosol. Those drugs which exert a moderate degree of antihistamine activity are: 929 F, 1571 F, 2325 R.P., Antergan, Benadryl and several quaternary nitrogen derivatives, Pyribenzamine, Hetramine, Antistine and several alkyl derivatives of β-2-biphenyloxyethyl-β-chloroethylamine (1). A remarkable degree of potency was exhibited by Neoantergan, alkamine derivatives of thiodiphenylamine (3015 R.P. and 3277 R.P.) and α-naphthylmethylethyl-β-chloroethylamine (1). 3277 R.P. prevented immediate deaths following intravenous injection of 1500 lethal doses of histamine (80) in guinea pigs, whereas epinephrine and Aleudrine were incapable of protecting against two lethal doses.

It is known that the relative potency of drugs based on reactions in a given organ or tissue may be decidedly different when based on reactions in other tissues or organs. Because of this fact it is highly important to ascertain the degree of effectiveness of antihistamine drugs with respect to the antagonism of the action of histamine on the vascular system, intestinal and uterine smooth muscle, etc. Unfortunately, there is a paucity of data referable to such comparisons (vide infra), which is regrettable since a demonstrable degree of selective activity in certain tissues is important when selecting drugs for experimental and clinical use.

With several antihistamine drugs it has been shown that histamine-induced bronchioconstriction was diminished in isolated, perfused, guinea-pig lungs (49, 169, 192) and a limited amount of evidence indicated diminution of the histamine effect in other animals (50, 192) and man (35). Hewitt and Curry (84) found that Benadryl ameliorated bronchioconstriction and systemic reactions to impure preparations of streptomycin which contain histamine or a similar substance.

2. *Intestinal muscle.* Staub (169) reported that 929 F and 1571 F in a dilution of 2×10^6 to 1×10^7 prevented histamine from contracting isolated guinea-pig ileum. There is agreement (31, 77, 112, 187) that sufficient doses of 929 F or 1571 F suppressed the spasmogenic action of acetylcholine and barium, thus indicating that these antihistamine compounds are not absolutely specific as was suggested for 929 F by Rosenthal and Brown (156). According to Halpern (77), the dilution of drugs required to completely suppress the spasmogenic action of histamine (3.3×10^6; base?) on guinea pig intestine was 6.6×10^4 for 1571 F, 6.6×10^5 for 2325 R.P. and 1×10^7 for Antergan. In order to antagonize the spasmogenic action of acetylcholine and barium it was necessary to increase the concentration of Antergan and 2325 R.P. several hundred times, the latter compound being more specific but one-fifteenth as potent as Antergan in antihistamine action.

Other direct comparisons of the antihistamine action of several drugs on intestinal muscle were made by Loew *et al.* (112) who found that spasm induced

with histamine diphosphate (1.25×10^7) was reduced 75 to 100 per cent by 1571 F in a dilution of 3.3×10^6 and by Benadryl in a dilution of 5×10^7, whereas antispasmodics such as atropine, Pavatrine, and Trasentin were only effective in lower dilutions of approximately 1×10^6. It was also revealed that Benadryl, and especially 1571 F, were less effective than Pavatrine and Trasentin as regards antagonism of the spasmogenic action of barium and acetylcholine. Thus, the most pronounced action of 1571 F and Benadryl was their antagonism to histamine, Benadryl being most potent and 1571 F the most specific. An extension of these studies (109, 187) with several antispasmodics, Benadryl and some of its quaternary derivatives, revealed interesting differences in potency and specificity relating to antagonism of histamine, barium and acetylcholine. Histamine-induced intestinal spasm was diminished by Antistine (127), a drug which exhibited a moderate degree of specificity.

Since Neoantergan is markedly effective in relieving histamine-induced bronchioconstriction, it is unfortunate that there are no quantitative and comparative data regarding its antihistamine action with reference to isolated intestinal muscle. It has been stated (42) that Benadryl was much less effective than Neoantergan and that the inhibiting dose of Neoantergan was less than the dose of histamine required to induce spasm (24). Mayer (118) stated that a dilution of Pyribenzamine of 5×10^7 was sufficient to prevent the spasmogenic action of histamine in a dilution of 1×10^7 and occasionally 1×10^6. Pyribenzamine (118) and Neoantergan (42) weakly antagonized the spasmogenic action of acetylcholine.

In anesthetized dogs, spasm of the intestine induced with histamine has been blocked by 929 F (169), Pyribenzamine, Neoantergan and Benadryl (167).

The antispasmodic action of antihistamine drugs as evidenced by their ability to diminish the tonus and spontaneous motility of isolated rabbit intestine, or the intestine of intact dogs, was not marked in the case of 929 F and 1571 F (76, 169), 2325 R.P. (77), Neoantergan and Pyribenzamine (42, 167). In fact, Neoantergan and Pyribenzamine stimulated intestinal and uterine activity when injected intravenously in anesthetized dogs, whereas Benadryl decreased tonus and motility of the intestine (167). In unanesthetized dogs, 929 F usually had only a variable and transient inhibitory action on the stomach, duodenum and jejunum and no important effect on the terminal part of the ileum (76).

In view of these findings it is quite apparent that the majority of antihistamine drugs do not prevent spasm or exert a prominent relaxing effect on intestinal muscle except under conditions in which histamine has produced increased tonus, hypermotility or spasm. Benadryl exerts a weak antispasmodic action.

3. *Uterine muscle.* Histamine has a powerful oxytocic action. Mayer (118) stated that 929 F and 1571 F were capable of contracting the isolated guinea-pig uterus, their spasmogenic action being sufficient to interfere with a demonstration of antihistamine activity. However, Staub (169) had previously reported that 929 F, in a dilution of 2×10^5, contracted the uterus whereas a dilution of 1×10^5 failed to do so and antagonized the oxytocic action of histamine. Mayer (118) stated that the antihistamine activity of Antergan and

Pyribenzamine was sufficiently higher than their spasmogenic activity to permit a demonstration of histamine antagonism. Halpern (77) reported that both Antergan and 2325 R.P. exerted a strong excitant action on isolated guinea-pig uterus. According to Bovet and Walthert (24), both Antergan and Neoantergan exhibited a degree of spasmogenic action which precluded a satisfactory demonstration of specific antihistamine action on the isolated guinea-pig uterus, although Dews and Graham (42) demonstrated histamine antagonism with Neoantergan in a dilution of 5×10^8; with rat uterus, the inhibiting action of histamine was not altered by non-spasmogenic amounts of Neoantergan. An obvious need exists for quantitative data obtained from several animal species to replace the statements, opinions and "typical" experiments which concern the spasmogenic action of 929 F, 1571 F, Antergan, 2325 R.P., Pyribenzamine and Neoantergan, and the ability of these drugs to antagonize the oxytocic action of histamine. No data are available concerning the action of Benadryl on the isolated uterus.

In anesthetized dogs, the intravenous injection of Pyribenzamine and Neoantegan in doses of 3.0 mgm. per kgm. caused contraction of the uterus, whereas the same doses of Benadryl failed to do so (167); each of the drugs antagonized the contracting effect of injected histamine.

The limited data available warrant the tentative conclusion that the antihistamine agents, with the exception of Benadryl, are capable of contracting uterine muscle, and that all antagonize the spasmogenic action of histamine. Although Benadryl exerted a weak antispasmodic action on bronchiolar and intestinal muscle, none has yet been experimentally demonstrated for uterine muscle. The reviewer is unaware of any evidence whatsoever concerning the ability of antihistamine drugs to oppose actions of pituitrin on the uterus or other tissues. Quantitative data relative to effects of antihistamine drugs on uterine tissue of several species of animals are needed, and studies should determine the effectiveness and specificity with regard to antagonism of various spasmogenic agents, especially pituitrin.

4. *Arterial blood pressure.* Although Staub (169) reported that 929 F failed to antagonize the depressor action of histamine in chloralosed dogs, positive results were later demonstrated (31, 132) when small doses of histamine were employed. In rabbits, histamine shock (178) and hypertension induced with small doses of histamine have been diminished by 929 F (169) and hypotension induced with large doses of histamine in unanesthetized rabbits was diminished by 1571 F, Antergan and Neoantergan, the latter drug being effective in intravenous doses of 0.5 to 1.0 mgm. per kgm. of body weight (143).

Reports by Bovet and co-workers (20, 21, 24) revealed that Neoantergan was markedly effective in diminishing the depressor action of histamine in anesthetized dogs; nasal plethysmograph measurements indicated lessened vasodilatation. Pyribenzamine (167, 189) and Benadryl (112, 167, 181, 182) also diminished the depressor action of histamine in dogs. Comparative studies with Neoantergan, Pyribenzamine and Benadryl in dogs anesthetized with pentobarbital (167) revealed that each drug in intravenous doses of 3.0 mgm. per

kgm. body weight diminished the depressor action of histamine the same degree (*ca.* 55 per cent). Thus, these drugs appeared to possess equal potency with regard to antagonizing the depressor effects of identical doses of histamine in dogs even though their ability to antagonize the bronchioconstrictive action of histamine in guinea pigs varied widely. However, examination of data relating to Benadryl-histamine antagonism (112, 182) reveals that the percentage inhibition of the depressor action of histamine only varied within small limits when the dose of Benadryl was varied several-fold. It is therefore possible that quantitative data derived from experiments of suitable design may reveal differences in potency of these drugs. Neoantergan and thiodiphenylamine derivatives (3015 R.P. and 3277 R.P.) prevented death of dogs, rabbits and guinea pigs following tremendous doses of histamine injected intravenously (80).

Benadryl produced some diminution in the depressor response to acetylcholine (112, 167, 168) and since it weakly antagonized the spasmogenic action of acetylcholine on intestinal muscle (108, 109, 112, 187) and was capable of producing mydriasis and altering accommodation (82, 109, 126), it is apparent that Benadryl has a weak atropine-like action. The evidence available indicates that 1571 F, Antergan, 2325 R.P., Neoantergan and Pyribenzamine have only a slight atropine-like action (24, 77, 169) on intestines and bronchioles and the latter two compounds fail to diminish the depressor action of acetylcholine (167).

The essential pharmacological difference between Benadryl and the α-aminopyridine derivatives appears to be that Benadryl is less specific as an antihistamine drug by virtue of a higher degree of anti-acetylcholine action which may be expressed as an antispasmodic effect not appreciably apparent with Neoantergan and Pyribenzamine. In fact, the latter are spasmogenic in certain concentrations. Quantitatively, Neoantergan and Pyribenzamine were more effective than Benadryl in diminishing severity of histamine shock in guinea pigs (65, 66, 167) but the data now available are not sufficient to prove that there is a wide difference in effectiveness in antagonizing actions of histamine on the vascular system. Since the clinical literature reveals very close similarity in the ability and inability of Benadryl and Pyribenzamine to alleviate symptoms of various allergic diseases, it is apparent that clinical effectiveness is not directly related to degree of antihistamine action in guinea pigs.

It may be concluded that antihistamine drugs not only antagonize excitatory effects of histamine on bronchiolar, intestinal and uterine smooth muscle, but also prevent the relaxing or inhibitory effects of histamine on the vascular system, as indicated by antagonism of the depressor action of histamine in dogs and its coronary dilating action (42) in cats.

The data now available relative to the effects of antihistamine drugs on bronchiolar, uterine and intestinal smooth muscle, indicate that the term musculotropic antispasmodic fails to characterize definitely drugs which are potent and appreciably specific as antagonists of the spasmogenic action of histamine. The term antispasmodic would not be applicable with reference to the ability of these drugs to prevent histamine from *relaxing* smooth muscle of systemic arterioles and coronary vessels in carnivores, or from increasing permeability

of capillary endothelium (vide infra). For these reasons it becomes apparent that the term "antihistamine" is most useful in describing and classifying these drugs.

5. *Capillary permeability and cutaneous reactions.* The question regarding the rôle which histamine plays in inflammatory reactions is important for such reactions are elicited by thermal, chemical, mechanical and photic stimuli; those reactions which are referable to bacteria or allergens are intimately related to immunological processes. In the localized inflammatory response, the flare and wheal are related, respectively, to vasodilatation and accumulation of edema fluid. The immediate cause of vasodilatation and especially of localized edema in inflammatory reactions is therefore of importance and increased capillary permeability is an important factor relative to the transudation of fluid from capillaries to injured tissues (97, 131).

Of the substances known to increase capillary permeability, histamine is probably the most potent (34, 102) and comparatively large amounts are present in skin (4, 10, 74, 116, 155, 157). Histamine appears to be somewhat specific in inducing cutaneous reactions for it was effective at a concentration of M/10,000 whereas a variety of other amines, imidazoles and miscellaneous substances were found to be effective only in concentrations of M/2 to M/100 (34).

The measurement of cutaneous responses to histamine, allergens, and other substances are semi-quantitative and usually restricted to observations, or actual measurement, of the diameter of the area showing a flare and wheal. The effects of antihistamine drugs on these cutaneous reactions have seldom been measured in a precise manner and the scope of the various studies has been limited. For these reasons, the discussion regarding the effect of antihistamine drugs on localized cutaneous reactions to a variety of stimuli is largely descriptive rather than explanatory.

In the rabbit and dog, extravasation of dye has been used as a measure of increased capillary permeability. Benadryl (98), Neoantergan (14, 15, 98) and Pyribenzamine (145) diminished or annulled the localized accumulation of dye which usually follows intradermal injection of histamine. Benadryl did not protect the cutaneous capillaries of the rabbit against other injected or liberated substances (98) since trypan blue responses to trypsin, venom of Crotalus adamanteus, staphylococcus toxin (Burky strain Ha), heparin, tetracaine (Pontocaine), codeine, and horse serum were unaltered in non-sensitized rabbits. Although Friedlaender and Feinberg (64) believed that local application of Benadryl reduced a wheal and flare response to codeine sulfate (scratch test) in man it is pertinent to consider that a 5 per cent solution of Benadryl would exert local anesthetic action and thus affect axon reflexes concerned in flare reactions. Data from sensitized rabbits were suggestive but did not prove or disprove that histamine was importantly involved in the cutaneous response to antigen (98). None of the evidence obtained or cited suggested that choline or an ester was concerned in the responses to any of the agents employed.

The experimental data obtained from rabbits (98) strongly suggest a, that the action of antihistamine drugs on capillary permeability was limited to an

antagonism of histamine, and b, that trypsin, snake venom, and staphylococcus toxin increased capillary permeability through some mechanism which did not involve histamine. The studies made with non-sensitized rabbits provided no support for the contention that intradermal injections of various agents causes liberation of histamine which accounts for increased capillary permeability (154), although similar studies with other antihistamine drugs should be made in several animal species.

The wheal and flare referable to intracutaneous injections of histamine in man were frequently diminished following oral administration of Antergan (28, 136, 139, 176, 179), Neoantergan (176), Benadryl (48, 57, 64, 126, 141) and Pyribenzamine (7). The highest incidence and degree of diminution of cutaneous responses to histamine occurred when threshold doses of histamine were injected in subjects treated with adequate doses of antihistamine drugs.

This evidence that antihistamine drugs diminish cutaneous reactions to administered histamine suggests that the drugs should diminish reactions to substances known or suspected of containing histamine. Trypan blue responses of equal intensity produced in dogs by histamine and ascitic fluid were attenuated proportionally by Neoantergan (15), thus suggesting that ascitic fluid contains histamine. Other evidence of the presence of histamine in ascitic fluid and pleural exudates has been adduced (12, 15, 152, 153). It is conceivable that some of the reactions observed by Menkin (129–131) in his studies on inflammation which were attributed to a specific factor, leucotaxine, may have been due to histamine.

More data are needed relative to the antagonism of the cutaneous effects of snake (136, 139) and bee venoms (2). It was stated that Antergan therapy had no effect on erythema induced by ultraviolet irradiation of a limited area of human skin (139).

Frequent attempts have been made to diminish localized anaphylactic reactions in the skin of animals and man by pretreatment with antihistamine drugs. Large doses of 929 F and Antergan in tuberculous guinea pigs did not minimize the severe cutaneous reactions produced by injection of 10 mgm. of crude tuberculin intradermally; Antergan failed to diminish cutaneous responses to smaller amounts of tuberculin (16). In man, the cutaneous responses to both histamine and tuberculin were diminished by Antergan (26).

Hemorrhagic cutaneous reactions (Schwartzman), which followed intravenous injections of bacterial toxin in rabbits previously sensitized locally, were diminished by Neoantergan administered subcutaneously in divided doses over a period of ten hours (24). Failure of 929 F and Antergan to diminish such hemorrhagic responses (17) in several rabbits could have been referable to the use of large doses of toxin and inadequate duration of treatment with drugs less potent than Neoantergan.

Support for the concept that liberation of histamine from tissues accounts in part for the allergic type of inflammatory reaction is the evidence that antihistamine drugs diminish dermographic responses (8, 57, 64, 136, 139, 163) in man and the responses induced by intradermal injection of allergens (7, 64,

176). In addition, the clinical literature reveals that a comparatively high percentage of patients with acute or chronic urticaria derive definite symptomatic relief when treated with antihistamine drugs. Diagnostic skin-testing procedures effected to determine the presence of hypersensitivity should obviously not be conducted in patients who have recently ingested an antihistamine drug.

Antihistamine drugs probably diminish whealing by preventing histamine from increasing capillary permeability or by acting directly to decrease permeability. If the action is direct, antihistamine drugs should diminish increased capillary permeability caused by a variety of agents which do not contain or liberate histamine. According to Gelvin, Elias and McGavack (70), Benadryl did not affect the permeability of meningeal capillaries which are permeable to Benadryl since comparable quantities of the drug were found in blood and spinal fluid of seven patients (71). According to the prevailing concept, the erythematous flare induced by cutaneous injury depends upon axon reflexes. Evidence obtained by Parrot and Lefebvre (138) indicated that Antergan prevented histamine from initiating such reflexes; the failure of Antergan to prevent vasodilatation in dogs following electrical stimulation of antidromic nerve fibers rendered it unlikely that histamine was acting as a mediator.

It will be important to determine the extent to which the action of antihistamine drugs is referable to a direct effect upon capillaries, an antagonism of the effect of histamine on permeability and ability to initiate or mediate axon reflexes, or an enhancement of the effects of epinephrine (cf. discussion concerning specificity). Antihistamine drugs which fail to enhance, or actually block, effects of epinephrine may prove useful in elucidating the effects upon capillaries.

Since antihistamine drugs were capable of diminishing the vasodepressor action of histamine and its action of increasing capillary permeability it is significant to note that Benadryl (85) and Antergan (88) failed to induce beneficial effects in hemorrhagic and traumatic shock, respectively.

6. *Glandular secretion.* The ability of antihistamine drugs to antagonize the actions of histamine on the vascular system and on smooth muscle in several locations suggests that such drugs might inhibit lacrimal, salivary, gastric and pancreatic secretion following stimulation of these glands with histamine. If so, they could be employed advantageously to elucidate the question as to whether histamine plays any physiological rôle in these secretory processes. Whether histamine is related to the secretory response of the stomach to food and the secretion occurring in abnormal conditions is a moot question (51, 73, 87). If hypersecretion and gastric retention are etiological or aggravating factors in "peptic ulcer," a drug with gastric secretory depressant and antispasmodic properties would certainly warrant clinical trial.

Loew and Chickering (106) found that subcutaneous injection of 929 F in dogs with gastric pouches (Heidenhain) previous to a subcutaneous injection of histamine actually increased the volume and acidity of secretion over that which obtained during control periods when only the histamine stimulus was employed. Hallenbeck (75) corroborated these findings in experiments in which the histamine stimulus was effected in a more physiological manner, viz.

by slow intravenous infusion or absorption from a beeswax mixture. By an unknown mechanism, 929 F did diminish secretion in response to a meal stimulus in dogs with innervated and denervated pouches (Pavlov, Heidenhain, transplanted).

In dogs with denervated pouches, 1571 F failed to modify secretion after a food stimulus (18) but inhibited secretion in dogs with innervated pouches (75), which suggests that the drug exerted a central nervous action. Ignorance of the fact that secretion may vary according to whether the gastric pouch is innervated or denervated undoubtedly accounts for the statement (146) that, "The effect of compounds 929 F and 1571 F on gastric responses to histamine or on (*sic*) food ingestion in dogs varies with the experimenters." 1571 F almost halved the output of histamine-induced secretion (18) although when large single doses of histamine were employed (27) neither 929 F nor 1571 F inhibited secretion. In summarizing, it is apparent that 929 F failed to antagonize the gastric secretogogue action of histamine and that secretory inhibition demonstrated with 1571 F was probably not referable to a specific antagonism of histamine since pilocarpine-induced secretion from denervated pouches was also inhibited (18).

Pretreatment of dogs with Benadryl inhibited secretion from denervated gastric pouches approximately 40 per cent when the histamine stimulus (0.5 mgm. histamine diphosphate) was given as a single subcutaneous injection (112). However, secretion was not inhibited in all dogs and recent evidence indicates that Benadryl (67, 160) and Pyribenzamine failed to antagonize the secretogogue action of histamine in dogs with various types of pouches. Some inhibition was noted after treatment with several quaternary nitrogen derivatives of Benadryl (69). Benadryl did not prevent formation of duodenal and gastric ulcers in dogs which received large amounts of histamine in beeswax (67). No conspicuous inhibition of histamine-induced gastric secretion occurred in dogs pretreated with diethylaminoethyl-dihydroanthracene (100), diethylaminoethyl fluorene-9-carboxalate (99) or several alkyloxytriazines (105) which weakly antagonized histamine (108). Thiodiphenylamine derivatives (3015 R.P. and 3277 R.P.) apparently failed to inhibit salivary, pancreatic and gastric secretion (80).

In the rat, Neoantergan did not inhibit the secretogogue action of histamine (50 mgm./kgm., subcutaneously), although atropine (200 mgm./kgm., subcutaneously) was effective (24). Seemingly the rat is a poor choice of experimental animal if such large doses of drugs are required to stimulate or depress secretion.

It was stated (164) that the previous ingestion of Antergan by human subjects did not modify secretion of acid following injection of histamine. In six subjects (37) the intravenous injection of Antergan in doses sufficient to produce definite symptoms reduced the volume but not the total amount of hydrochloric acid secreted following subcutaneous injection of histamine dihydrochloride (1.0 mgm.), although the local and systemic effects were attenuated. Neoantergan also failed to reduce the total acid secreted by two subjects, although it eliminated all other effects of histamine. Preliminary studies in man did not provide

unequivocal evidence that Benadryl appreciably antagonized the secretogogue action of histamine (123, 124) and of alcohol (125), which may liberate histamine. Appreciable doses of Benadryl infused intravenously failed to significantly decrease the volume and free acid concentration of gastric juice in duodenal ulcer patients given a test meal, or histamine, subcutaneously or intravenously (133).

The available evidence from man and animals justifies the conclusion that none of the antihistamine drugs directly antagonized the gastric secretogogue action of histamine. Thus, at least one of the prominent effects of histamine was not significantly diminished by antihistamine drugs.

Halpern (77) has stated that the intravenous injection of 2325 R.P. and Antergan in dogs augmented salivary secretion and that thiodiphenylamines (80) did not affect salivary, pancreatic and gastric secretion. In anesthetized cats, Yonkman *et al.* (190) noted that salivary secretion induced by carotid injections of histamine was highly variable but appeared to be diminished or annulled by Pyribenzamine. Lacrimation induced with histamine was stated to be definitely suppressed after injections of Pyribenzamine. Salivary secretion was still elicited by faradization of the chorda tympani nerve or by injecting pilocarpine. In some experiments, Pyribenzamine appeared to sensitize the salivary secretory response to such a variety of agents and procedures as to suggest that experimental conditions were improperly controlled. Among patients receiving therapeutic doses of Antergan, Pyribenzamine or Benadryl there is such a low incidence of xerostomia (163, 146) as to suggest that neither the antihistamine nor atropine-like qualities of these agents are physiologically important in relation to salivary secretion.

Because of the absence or paucity of data and the non-quantitative nature of that which exists, it would be premature to express an opinion concerning the ability of antihistamine drugs to antagonize the secretogogue action of histamine on lacrimal, salivary and pancreatic glands. Since histamine-induced gastric secretion was not antagonized, consideration must be given to the possibility that histamine acts on secretory cells through some mechanism which differs from that obtaining in endothelial and smooth muscle cells; or, antihistamine drugs do not reach or have no affinity for the elements in the secretory cell which react to histamine.

EFFECT OF ANTIHISTAMINE DRUGS IN ANAPHYLAXIS AND ALLERGY. It is not the prime purpose of this review to present a detailed, critical evaluation of the evidence relating to the histamine theory of anaphylaxis and allergy (cf. 10, 32, 44, 45, 60, 149). The problem is of extreme importance, however, both from theoretical and practical standpoints. Therefore, at least a portion of pertinent evidence now available relative to the use of antihistamine drugs in elucidating the problem is cited.

The symptoms and reactions to histamine in a given species are nearly identical to those occurring in anaphylaxis. The reactions to histamine differ from one species to another since different shock organs or tissues give rise to the dominant reactions. Correspondingly, the anaphylactic reactions differ from

species to species, but in a given species they are always strikingly similar to histamine reactions. Antihistamine drugs should therefore be effective in diminishing histamine and anaphylactic reactions in each species if histamine is significantly involved in anaphylaxis.

All antihistamine drugs which so effectively antagonize the bronchioconstrictive action of histamine are capable of diminishing the severity of anaphylaxis in the guinea pig, in which animal bronchioconstriction is the prominent feature (for refs. cf. 32, 44, 60, 183) of anaphylaxis. Anaphylaxis has been alleviated in intact guinea pigs by treating with 929 F (156, 169, 170), 1571 F (109, 169, 186), Antergan and 2325 R.P. (77, 103), Neoantergan (24), Benadryl (65, 107, 165), Pyribenzamine (7, 65, 118, 122), Antistine (127), Hetramine (59), the thiodiphenylamine, 3015 R.P. (79), and α-naphthylmethylethyl-β-chloroethylamine (1). Tolerated doses of antihistamine drugs are more effective in the prevention of both histamine and anaphylactic shock than comparatively large and even poorly tolerated doses of atropine (77), theophylline (52, 100, 107, 110, 111), papaverine (62, 107, 110, 111, 169) epinephrine (77, 90, 100, 111) and other drugs possessing antispasmodic action (77, 100, 142, 169). All of this evidence, and especially that obtained with antihistamine drugs, supports the belief that histamine is involved in producing the symptoms of anaphylaxis.

With antihistamine drugs, the lowest doses (rarely proven to constitute minimal effective doses) used to demonstrate anti-anaphylactic action in guinea pigs are sometimes greater than the minimal effective doses required to relieve histamine shock. For example, the doses of Pyribenzamine, Neoantergan and especially 3015 R.P. used to demonstrate anti-anaphylactic action (24, 65, 79) were appreciably greater than one would expect necessary in view of their effectiveness in antagonizing administered histamine. Before this type of data can be used as evidence that factors other than histamine are importantly involved in anaphylaxis it will be necessary to obtain quantitative data relating to anaphylactic and histamine shock under conditions as strictly comparable as possible. If the protective action of antihistamine drugs is satisfactorily demonstrated to be appreciably less against anaphylactic shock than against histamine shock it will still not constitute weighty evidence that some factor(s) other than histamine is involved to an important degree since in anaphylaxis histamine may be released in more intimate contact with the effector portion of the reacting cells (100) than can be effected by administering histamine.

Anaphylaxis in the dog is intimately connected with the release and action of histamine (for refs. cf. 32, 44, 60, 183), the most conspicuous feature being hypotension resulting from vasodilatation, increased capillary permeability, contraction of hepatic veins and engorgement of tissues drained by the portal system. In a limited number of experiments, Antergan (77) and Pyribenzamine (191) diminished hypotension and symptoms of anaphylaxis in dogs. Wells, Morris and Dragstedt (183) demonstrated absence of mortality upon injection of horse serum in 22 sensitized dogs treated with Benadryl, whereas 9 or 34.6 per cent of the 26 untreated animals died. The persistence of some degree of hypotension when shock was induced in Benadryl-treated animals does not prove that agents other than histamine were involved since histamine blood

levels were increased and it was previously shown (182) that Benadryl diminished but did not abolish the hypotensive action of the quantities of histamine involved.

In rabbits, anaphylaxis or histamine administration causes transient hypertension and then hypotension and death due to circulatory failure which is secondary to a marked constriction of pulmonary blood vessels (44). Atropine, epinephrine and ephedrine (177) failed to diminish the hypotension occurring during anaphylaxis. Neoantergan (24, 143) proved more effective than 929 F, 1571 F and Antergan in diminishing the hypertensive responses to histamine in non-anesthetized rabbits. In anaphylaxis, 929 F (178) and the sympatholytic agents, 933 F and 883 F, were somewhat effective in diminishing reactions (174, 175) although appreciable vasodilatation and hypotension induced with these agents (41) may account, in part, for the effects. Additional data regarding the effectiveness of potent antihistamine agents, and other drugs, in controlling anaphylaxis in rabbits are needed. Comparisons with the effectiveness of aminophylline and a study of the mechanisms involved would be valuable in view of the possibility that the effectiveness of aminophylline in "epinephrine-fast" asthmatic patients may be due to its ability to diminish pulmonary vascular resistance (100).

Following publication of the statement that Neoantergan actually increased the lethal action of histamine in mice (24 and cf. 80), Mayer and Brousseau (119) compared the effects of other antihistamine drugs in anaphylactic shock and histamine shock. Although Pyribenzamine reduced the severity of anaphylaxis produced with large intravenous doses of antigen, these authors stated that Pyribenzamine and Benadryl were both definite *synergists* of histamine in mice. However, it appears probable that the synergism, or additive effect, was actually between the antihistamine drugs and the large volumes of acidic solution injected intravenously into such small animals (0.25 to 0.75 ml. of 2.0 per cent histamine acid phosphate per mouse; 1.0 ml. per minute). These results cannot be accepted as proof of an exception to the parallelism between histamine and anaphylactic shock in a given animal species.

Further evidence concerning the effectiveness of antihistamine drugs in diminishing or preventing anaphylactic responses is included in the section dealing with localized cutaneous reactions to histamine and antigens (vide supra) in animals and man.

A few studies relative to anaphylaxis *in vitro* have been made. The spasm of sensitized smooth muscle which follows addition of antigen to the perfusing or bath fluid is likely due to histamine liberated from the tissue. For the most part the reactions are qualitatively and quantitatively typical of those induced by adding proper amounts of histamine.

Bronchospasm induced by adding antigen to fluid perfused through isolated, sensitized, guinea-pig lungs was lessened by Benadryl (49), whereas Pyribenzamine (192) failed to decrease spasm, possibly because large amounts of antigen (10 mgm. of horse serum) were employed. Perfusion experiments with isolated lungs are not ideally suited for quantitative determinations of the relative potencies of antihistamine drugs or for determination of quantitative relation-

ships referable to degree of effect against antigen and histamine. 1571 F (186) prevented antigen and histamine from decreasing the flow through coronary vessels of the sensitized guinea pig-heart.

The spasm induced by adding antigen to isolated intestinal and uterine muscle from sensitized animals has been diminished or annulled by 929 F (156, 169, 170), Antergan and 2325 R.P. (77), 3015 R.P. (79) and Antistine (127).

Reactions to trypsin. In view of the similarity of reactions in trypsin shock and anaphylaxis, as well as the numerous observations which supported the hypothesis that the toxicity of trypsin was referable to the enzymatic release of histamine from tissues, it is significant that Benadryl 1, failed to provide protection against the lethal action of trypsin in guinea pigs and dogs; 2, only very slightly diminished the hypotensive action of trypsin in dogs (184), and 3, did not prevent the increased capillary permeability induced with crystalline trypsin in rabbit's skin (98). Furthermore, trypsin injections in dogs did not increase blood histamine sufficiently to account for severity of reactions (184), and in view of other evidence (171) it is now doubtful that the histamine hypothesis, relative to the toxicity of trypsin, is tenable.

Summary. Thus the evidence cited indicates that specific antihistamine drugs diminish several pharmacological responses to administered histamine which are of the type which could account for a variety of anaphylactic reactions. These drugs also diminish those anaphylactic responses which are considered as being due to histamine. Furthermore, in several animal species a parallelism exists between histamine and anaphylactic responses, both of which are prevented or controlled with antihistamine drugs. The use of specific antihistamine drugs as pharmacological tools has therefore provided evidence which even more firmly establishes the concept that histamine causes the major manifestations of anaphylaxis.

In addition, the effectiveness of antihistamine drugs in controlling certain allergic manifestations in man has been established and it is pertinent to note that therapeutic efficacy demonstrated thus far is restricted to allergic diseases, and possibly a few other conditions in which there are some reasons for considering histamine as an etiological agent. This constitutes strong evidence that histamine is involved to an appreciable extent in several allergic diseases.

Antihistamine drugs have been selected and partially evaluated on the basis of their striking effectiveness in alleviating bronchioconstriction induced in guinea pigs with histamine or by anaphylaxis. In clinical use these agents have exhibited much less efficacy in bronchial asthma than in angioneurotic edema, urticaria and seasonal rhinitis, i.e., in conditions in which vascular reactions undoubtedly play a prominent rôle. Limitation of space does not permit discussion of possible explanations of such differences, although mention should be made of the possibility that bronchiolar smooth muscle of the guinea pig differs markedly from that in other species, both in regard to sensitivity to histamine and the blocking action of antihistamine drugs.

Since antihistamine drugs do not always completely block effects of histamine and fail to diminish significantly certain effects such as the stimulant action of

histamine on gastric secretory cells, their failure to diminish or prevent a given response does not necessarily eliminate the possibility that histamine bears a causal relationship.

SPECIFICITY OF ANTIHISTAMINE DRUGS AND RELATION TO AUTONOMIC FUNCTIONS. From the experimental evidence available it appears that 1571 F, Antergan, Neoantergan, Pyribenzamine and Antistine exert a low, and probably insignificant, degree of atropine-like action. It is doubtful whether the antihistamine and anti-allergic activity of Benadryl is closely related to atropine-like action since such action is weaker (112, 187) than that exerted by antispasmodic drugs such as Pavatrine and Trasentin which do not prevent anaphylaxis or effects of histamine on bronchioles and the vascular system. In support of the belief that atropine-like action of antihistamine drugs is unimportant is the fact that those drugs employed clinically seldom produce tachycardia or inhibit salivation. From a theoretical standpoint, even a slight degree of atropine-like activity could be important if the drugs simultaneously enhanced activity of tissues receiving sympathetic innervation. Consideration must therefore be given to evidence that antihistamine drugs potentiate responses to epinephrine and stimulation of adrenergic nerves.

Antergan enhanced retraction of the nictitating membrane in rabbits and the rise of blood pressure in dogs following injections of epinephrine (40, 137). When adequate doses were injected intravenously, Benadryl, Neoantergan and Pyribenzamine increased the height and duration of the pressor response to epinephrine in anesthetized dogs (112, 167, 168, 189). Sherrod *et al.* (167, 168) demonstrated that this phenomenon was not referable to an atropine-like blocking action of the antihistamine drugs on the cardiac vagus since under the experimental conditions even atropine failed to enhance the pressor action of epinephrine, and furthermore, the antihistamine drugs still induced the phenomenon in atropinized and vagotomized dogs. The biological significance of this potentiation of epinephrine must be questioned until the phenomenon is demonstrated in unanesthetized animals or man and data are adduced to show that other adrenergic responses are appreciably augmented by antihistamine drugs, and that this property is significantly related to antihistamine action. The failure of Benadryl to elevate the blood sugar level in man or to decrease glucose tolerance (126, 144) strongly suggests that no appreciable sympathomimetic action was exerted since a sensitive indicator of epinephrine action is a rise in blood sugar (11). In cats, Pyribenzamine (189, 190) did not consistently enhance several responses to injected epinephrine and sympathetic nerve stimulation. No potentiation of adrenergic responses has been reported to occur after administration of 929 F, 1571 F, and 2325 R.P.

There is still other evidence that adrenergic potentiation or sympathomimetic action does not account for the pronounced antagonism of histamine effected by the drugs under consideration. There is agreement (77, 86, 111) that ephedrine fails to reduce the severity of bronchioconstriction induced with large doses of histamine in guinea pigs, although it did relieve bronchioconstriction in an asthmatic patient injected with histamine (35). Doses of epinephrine which

produce symptoms in guinea pigs are not as effective as tolerated doses of several antihistamine drugs in alleviating symptoms of severe histamine shock (77, 80, 111). The author was unable to demonstrate any synergism between ephedrine and Benadryl in reducing mortality of guinea pigs upon induction of histamine shock (105). A strong argument against the hypothesis that sympathomimetic properties, or some type of adrenergic potentiation, is a requirement for potent antihistamine action resides in the fact that 929 F antagonizes histamine as well as certain effects of epinephrine (22, 31); also there is the recent demonstration (1) that certain adrenergic blocking agents are also exceedingly potent antagonists of histamine, both actions being rapid in onset and of remarkable duration.

α-Naphthylmethylethyl-β-chloroethylamine diminished or blocked adrenergic excitatory actions induced by nerve stimulation or epinephrine injection in dogs and cats. Pressor responses to epinephrine were readily reversed. On a dosage basis, this naphthyl derivative was more effective than epinephrine in preventing histamine shock and *anaphylactic shock* in guinea pigs. In dogs, the hypotensive action of histamine was blocked or diminished. The antihistamine action could not be entirely dependent on sympatholytic or adrenergic blocking action since among higher alkyl homologues of the naphthyl compound there were individual compounds with singular sympatholytic potency which did not antagonize histamine. Furthermore, the author (105) has been unable to demonstrate antagonism of histamine with adrenergic blocking agents such as Priscol (83), dibenzyl-β-chloroethylamine (Dibenamine) (134) and a series of benzhydrylalkyl-β-chloroethylamines (113). The dioxane derivatives, 2-piperidinomethyl-1, 4-benzodioxane (933 F) and 2-diethylaminomethyl-1, 4-benzodioxane (883 F), which are best known as adrenergic blocking drugs, were somewhat effective in diminishing effects of injected histamine, as well as anaphylaxis, in rabbits (174, 175). Such effects could be related to vasodilatation and hypotension produced by these compounds (41). 933 F and 883 F failed to decrease severity of histamine shock in guinea pigs (105), although 933 F diminished histamine-induced intestinal spasm (173). If some potent antihistamine drugs actually do potentiate adrenergic responses whereas others exert a sympatholytic action, it is difficult to conceive that strong antihistamine action is dependent on either type of activity.

From the evidence now available it appears unlikely that the anti-anaphylactic and anti-allergic activity of Antergan, Neoantergan, Benadryl, Pyribenzamine and Antistine is importantly related to either an atropine-like activity or to an augmentation of adrenergic responses. These drugs can be regarded as fairly specific antagonists of histamine. Further studies relating to specificity of action are needed and the detailed study of all antihistamine compounds should obviously include determination of atropine-like, sympathomimetic, sympatholytic, quinidine-like, local anesthetic, antispasmodic and other properties.

Mode of action. Mode of drug antagonism may be of three types (30, 68, 148). An example of indirect physiological antagonism is the ability of epinephrine to induce biological responses which are independent of, and dia-

metrically opposed to, such actions of histamine as the production of bronchospasm, vasodilatation, increased capillary permeability, contraction of intestinal muscle, etc. This type of antagonism is rather non-specific in character since epinephrine will diminish or annul certain pharmacological effects of numerous drugs. The other two types are both direct antagonisms. Drug action may be annulled or diminished if direct chemical reactions render the drug inactive by reduction, oxidation, conjugation or formation of salts or complexes. Finally, a direct antagonism may involve competition between two substances for a given site of action or receptive substance. This type of antagonism is reversible and one of the most specific in nature.

It has been repeatedly stated that there is no indication that antihistamine drugs act by combining with or destroying histamine, or by activating diamine oxidase or "histaminase" which are known to inactivate histamine and other amines, although the only pertinent evidence reported concerns 929 F (31, 169).

The major action of antihistamine drugs is probably exerted directly on the peripheral effector cells which respond to histamine since antagonism has frequently been demonstrated on isolated tissues or organs. The antagonism exerted peripherally could be modified by nervous or humoral influences in the intact animal. Quantitative data obtained by Wells *et al.* (181, 182) indicates the probability that Benadryl antagonizes the hypotensive action of histamine in dogs by adsorbing onto the site of action of histamine where it exerts no action except that of competing with and diminishing the action of histamine. The quantitative data reported by Halpern (81) concerning Antergan-histamine antagonism on isolated intestinal tissue also renders it probable that a direct, competitive inhibition was involved. The available evidence suggests that the antagonism of histamine is comparable to the antagonism between atropine and acetylcholine, and between adrenergic blocking agents and epinephrine, where there is a competition between two types of molecules (30, 148, 182).

Although antagonism of histamine with drugs may be regarded as a competitive affair it does not necessarily involve compounds with close chemical structure as is the case with chemical analogues in the p-aminobenzoic acid-sulfonamide and other metabolite-antimetabolite systems (cf. 180). Quaternary ammonium derivatives of Benadryl are choline ethers which possess, in addition to antihistamine action, the unique property of antagonizing acetylcholine (109, 112, 187). It is doubtful whether this choline ether-acetylcholine antagonism is closely related to analogous chemical structures since the most pronounced action was the choline ether-histamine antagonism where no similarity in structure obtains. Since Bovet and Walthert (24) first noted the diversity of the chemical nature of antihistamine drugs and the homogeneity of their pharmacological actions, several other types of antihistamine drugs have been found (1, 80, 110). The diverse chemical nature of compounds which antagonize histamine, along with the evidence that some of these compounds also alter responses to acetylcholine and epinephrine, immediately suggests that their antihistamine action is not closely related to a competition between chemical analogues. It would appear much more likely that antihistamine drugs alter surface tension or

are adsorbed onto one or more components of the reactive tissue so as to obviate the usual response to histamine. The specificity of antihistamine action varies with different compounds for some alter the response to acetylcholine, epinephrine and possibly other substances.

Rocha e Silva (151) pointed out the fact that there is a possible interdependence between the sites of attack of acetylcholine and histamine, since adequate quantities of atropine, the specific inhibitor of acetylcholine, also antagonize some of the effects of histamine (cf. 33). Other facts support the concept that acetylcholine and histamine act on closely related cellular components or on identical components in a slightly different manner. First, there is the close similarity of action of histamine and acetylcholine such as their secretory and vasodilator effects and the spasmogenic action on uterine, bronchiolar and intestinal smooth muscle. Secondly, some anti-acetylcholine activity is exhibited by most of the agents which antagonize actions of histamine, including the antihistamine drugs herein discussed, amino-acids (cf. 78, 142, 150, 151), certain derivatives of histamine and amino-acids (20, 150, 151), and a number of antispasmodics (52, 100, 108, 112, 161).

Consideration should probably also be given to an interdependence between the sites of attack of histamine and epinephrine for there are some similarities in their action, as well as conspicuous differences. It should be noted, however, that most of the antihistamine drugs, under certain conditions and with proper doses, either enhance or diminish certain effects of epinephrine. The three agents, acetylcholine, epinephrine, and histamine, all occur in the animal organism and may exert their physiological activity by acting through closely related mechanisms.

Some effects of epinephrine are diminished or blocked by 929 F (22, 31). Dioxane derivatives (883 F and 933 F) which block or reverse certain actions of epinephrine (41, and cf. 72) may antagonize histamine (169, 173–175), although the evidence is not entirely convincing. Of especial significance is the recent demonstration that α-naphthylmethylethyl-β-chloroethylamine, and related compounds, strongly antagonize certain actions of both histamine and epinephrine (1). Elucidation of the pharmacological properties and mode of action of compounds which antagonize both histamine and epinephrine in an extraordinary manner will indeed be important since comparisons can be made with results obtained with those antihistamine compounds (Antergan, Neoantergan, Benadryl and Pyribenzamine) which, under experimental conditions at least, are known to enhance the pressor and possibly other actions of epinephrine.

The effectiveness of antihistamine drugs in controlling anaphylaxis and allergy appears to be definitely related to their ability to diminish or block the effects of histamine upon vascular and visceral smooth muscle and permeability of capillaries. None of the evidence suggests that antihistamine drugs influence the antigen-antibody reaction. It is pertinent to note that blood levels of histamine increased in the usual manner following injection of antigen in dogs protected with Benadryl (183). The rise in blood histamine reported as occurring in normal human subjects following treatment with Antergan and Neoanter-

gan (140) requires confirmation and explanation. In animals, Antistine (128) and Antergan (104) did not inhibit antibody formation. The injection of Pyribenzamine (7) failed to change the complement titer of serum, and when the drug was mixed with antibody and antigen the precipitin titer was not altered.

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THE METABOLISM OF ACETIC ACID IN ANIMAL TISSUES

KONRAD BLOCH

Department of Biochemistry and Institute of Radiobiology and Biophysics, University of Chicago

Although acetic acid has long been suspected to be an intermediate in animal metabolism, the significance of its rôle in biochemical processes has only recently been recognized. The lack of interest in acetic acid metabolism during the past is in part attributable to the inadequacy of methods for identification and for quantitative determination of small amounts of acetic acid, and partly due to the fact that in isolated tissues acetic acid appears to possess only limited reactivity.

Until recently the principal approach to problems of intermediary metabolism has been the balance experiment, i.e., the measurement of the changes in concentration of the reaction product induced by varying concentrations of suspected precursor. The only reactions of acetic acid in animal tissues which could be reasonably well established by balance experiments were the acetylation of foreign amines (1, 2) and the formation of acetoacetic acid (3, 4, 5). A much greater variety of reactions involving acetic acid has been revealed with the aid of isotopically labeled substrates. The utility of the tracer technique for the study of intermediary metabolism derives from the fact that with its aid biochemical conversions become demonstrable irrespective of whether or not the total quantity of the reaction product undergoes a change. Thus the use of labeled acetic acid has been of particular service in detecting acetate formation and in demonstrating its participation in processes which proceed independently of exogenous acetate supply, viz., the biological syntheses of steroids, fatty acids, porphyrin, glucose and uric acid.

In general, it will prove more useful to review a broad segment of intermediary metabolism rather than the metabolism of a single compound. Indeed, the great diversity of biochemical processes, catabolic and anabolic, in which acetic acid is involved would require a much more comprehensive discussion of related subjects of intermediary metabolism than can be given here. Also, the present review is concerned mainly with the metabolism of acetic acid in animal tissues, although reference will be made frequently to processes occurring in other cells. This will be necessary because in many cases the discovery of a biochemical process in microorganisms has preceded and forecast an analogous reaction in animal tissues.

There is evidence to suggest that there exists a biologically active form of acetic acid but its identity has not been established. In the present review the terms acetic acid, acetyl and C_2 unit will be employed, sometimes interchangeably, without any intent of specifying a chemically defined entity.

THE ACETYLATION REACTION. The acetylation of foreign amines and amino acids will be discussed in some detail because this reaction has been of great value for the elucidation of various aspects of acetic acid metabolism in animal tissues.

Acetylation is one of the processes occurring with foreign amines prior to their elimination from the animal body. To what extent acetylation takes place in a given case will depend on the chemical nature and the dosage (6) of the foreign substance and on the species of the test animal. In rats, sulfanilamide is in part excreted unchanged, partly converted to N-acetyl sulfanilamide and oxidized to a hydroxyl compound which can subsequently be conjugated with glucuronic acid. No acetylation of aromatic amines such as sulfanilamide occurs in the dog (7, 13). Also, no unique mechanism exists for the disposal of foreign α-amino acids of the type represented by γ-phenyl α-aminobutyric acid or cyclohexyl alanine. Depending on experimental conditions, the feeding of these amino acids will yield varying proportions of N-acetyl amino acid, hydroxy acid, keto acid and oxidation products derived from the latter (8). Reactions of this kind have been classified as detoxications, a pharmacological term which is generally taken to mean that the body tends to modify foreign substances in such a manner as to render them less injurious to the cell either by increasing their solubility in biological fluids, thus preventing deposition of foreign bodies, or by conversion to derivatives which have less affinity for enzyme systems. The observation that in some instances the toxicity and insolubility of the conversion product exceeds that of the original compound, as in the case of acetylsulfanilamide (9), has made it evident that this type of change undergone by the foreign substance in animal tissues could no longer be adequately described as a detoxication (10, 11). Available evidence indicates that there are but few organic compounds which are entirely refractory to attack by animal cells. The fact that substances are metabolized which are not normally constituents of animal tissues does not necessarily imply the existence of a multitude of enzymes which operate only when the foreign substance is offered to the cell. Thus the coupling of glucuronic acid not only with camphor, borneol or phenol, but also with normally occurring steroid alcohols, or the acetylation of natural as well as of foreign amino acids suggests that these conjugation reactions are not induced by the administration of foreign substances but merely reflect events which are part of the normal intermediary metabolism. The acetylation of a foreign amine may then be attributed to an enzyme which has as its normal function the acetylation of a naturally occurring amine. The enzymes concerned in "detoxication" reactions have not been sufficiently characterized to answer the question whether there exist normal substrates for the enzymes which act upon foreign substances. The enzyme which catalyzes the acetylation of sulfanilamide has been purified (2), but the investigation of substrate specificity has not been extended to normally occurring amino acids. p-amino benzoic acid is acetylated by the same system although at a somewhat slower rate than sulfanilamide.

As an example of a purified enzyme which does not differentiate between a normal and foreign substrate, the l-amino acid oxidase of Green et al. (12) may be mentioned. In the presence of the enzyme, phenylaminobutyric acid, a foreign amino acid, is oxidatively deaminated more rapidly than any of the natural amino acids. Evidently, in this case enzymatic activity is determined

by the α amino acid configuration and not by whether the amino acid is foreign to the cell.

The view that metabolic occurrences with foreign substances in animal tissues mirror normal events formed the basis for the classical investigations of Neubauer, Knoop and Dakin, who employed phenyl-substituted compounds as models for the study of fatty acids and amino acid metabolism. It attests to the validity of the original concepts that the conclusions arrived at by these investigators some forty years ago have not required any fundamental revision.

Acetylation of amines. Following the early finding of Cohn (13) that p-nitrobenzaldehyde was converted by the dog to acetyl p-aminobenzoic acid, acetylation was more closely investigated with the phenyl derivatives of glycine and of α-aminobutyric acid (8, 14). Although these experiments were designed to study synthesis and degradation of amino acids, they yielded equally important evidence with regard to the participation of acetic acid in intermediary metabolism. Knoop (8) showed that the feeding of racemic phenylaminobutyric acid led to the excretion of an optically active acetyl derivative which he found to be identical with the acetyl compound formed in the animal from γ-phenyl α-ketobutyric acid. Knoop erroneously assigned the unnatural configuration to this acetyl amino acid and concluded that the acid of natural configuration had been converted to the acetyl derivative of the unnatural isomer. Similarly the acetyl derivative of what Knoop believed to be l-phenylalanine was excreted unchanged while the acetyl derivative of the amino acid of opposite configuration, judged from the behavior of the racemic acetyl amino aid, appeared to be readily metabolized (15). In view of these findings Knoop was unable to retain his original suggestion that acetylation was a process of normal amino acid metabolism. However, when the configuration of the stereoisomers of phenylalanine was established by du Vigneaud and Meyer (16) it became clear that the configurations assigned by Knoop were incorrect and that, as was subsequently shown by du Vigneaud and Irish (17), the acetylphenylalanine which was metabolically inert belonged to the d-series. The same authors demonstrated that the acetyl compound excreted after feeding of racemic phenylaminobutyric acid was derived from the l-acid and that it was the amino acid of unnatural configuration which underwent optical inversion. These findings re-established the experimental basis for the acetylation hypothesis. Knoop had originally suggested that acetylation was part of a synthetic process leading to acetylamino acids by condensation of the corresponding keto acids with pyruvic acid and ammonia, in analogy to the in vitro reactions described by Erlenmeyer (18) and deJong (19). That acetylation was not necessarily linked to the amination of keto acids was shown by du Vigneaud et al. (20), who found that phenylamino butyric acid labelled with N^{15} retained during its conversion to the N-acetyl derivative nearly all of the N^{15} originally present, indicating that acetylation of the amino or imino acid had occurred. On the other hand, N^{15} was entirely replaced by normal nitrogen when similarly labeled d-phenylaminobutyric was converted to the acetyl l-acid, a result which pointed to the intermediate formation of phenylketobutyric acid. No need exists to postulate two separate

mechanisms for the acetylation of the isomeric amino acids if it is assumed that acetylation is preceded by inversion of the d- to the l-acid. The same authors also studied the acetylation of phenylaminobutyric acid in animals whose body fluids were enriched with D_2O. One atom of deuterium was introduced at the α carbon atom during the acetylation of both isomeric phenylaminobutyric acids. This can be explained readily in the case of the d-acid as resulting from the reductive amination of the intermediate keto acid by hydrogen derived from the labeled body fluids. On the other hand, introduction of deuterium at the α carbon atom of the acetyl l-acid without simultaneous detachment of nitrogen, as shown by the retention of N^{15}, can be visualized only if there exists an equilibrium between amino and imino acid which permits reversible hydrogenation and dehydrogenation. du Vigneaud et al. (20) therefore suggested a mechanism of acetylation in which condensation of the imino acid with pyruvic acid was the initial step, a scheme akin to that of Knoop (8) with the difference that the imino acid takes the place of keto acid plus ammonia. The formulation of the reaction was based on the assumption that pyruvic acid was the most likely source of acetyl. The subsequent demonstration that acetic acid is an acetylating agent for amines (1, 21) eliminated the necessity to associate the reversible hydrogenation-dehydrogenation, as indicated by the uptake of isotopic hydrogen at the α carbon of acetyl amino acids, with the acetylation reaction. It is more likely that amino-imino acid equilibria are established independently of the acetylation reaction as a general property of amino acids. In some instances the acetylation of foreign amino acids of unnatural configuration seems to proceed more rapidly than oxidative deamination and subsequent inversion. S-benzyl d-cysteine (22), p-bromophenyl- and p-bromobenzyl d-cysteine (23) are in part converted to acetyl d-amino acids. These results provide additional evidence that acetylation can be independent of the amination of keto acids.

Precursors of acetyl groups. From the many attempts to identify the nature of the acetyl precursor by balance experiments with intact animals no clear picture has emerged. For instance, there are reports both that the dietary addition of acetate depresses (24, 25) and increases (26) acetylation of sulfanilamide. In balance experiments with intact animals a significant answer is to be expected only if the exogenous supply of precursor is the limiting factor. The probable acetyl precursors such as acetate, pyruvate, or acetoacetate must be available from metabolic reactions in much greater quantities than are required for the acetylation of the relatively small amounts of foreign amine which can be given to animals so that the dietary addition of precursors is unlikely to influence the yield of acetylated product. Direct evidence concerning the sources of acetyl groups for foreign amines has been obtained from feeding experiments with isotopically labeled test substances. Bernhard has administered to humans and rabbits deuterio acetic acid (21) and deuterio ethanol (27), as a source of acetyl for either sulfanilamide, p-amino benzoic acid or cyclohexylalanine (28). In all cases high concentrations of deuterium were found in the excreted acetyl derivatives, demonstrating the conversion of both acetate and ethanol to acetyl groups for aromatic amines and foreign α amino acids. Similar

results were obtained with the stereoisomers of phenylaminobutyric acid by Bloch and Rittenberg (29, 30), who studied the quantitative aspects of the acetylation reaction. When equal amounts of labeled acetate are fed together with either d- or l-phenylaminobutyric acid, or with sulfanilamide or p-aminobenzoic acid, the excreted acetyl groups contain in all cases about the same concentrations of isotope. This indicates that acetic acid is an equally efficient source of acetyl groups for the two types of foreign amines represented by phenylaminobutyric acid (α amino acid) and sulfanilamide (aromatic amine). From the data obtained it was concluded that acetic acid is the main source of acyl groups in the acetylation of foreign amines. The level of isotope in the excreted acetyl groups amounts to a relatively small fraction only of the labeled acetic acid administered. This dilution of isotope is believed to result from the mixing of the exogenous labeled acetate with acetic acid arising in intermediary metabolism (30).

The ability of acetic acid to acetylate amines is also clearly indicated by experiments with surviving tissues and tissue extracts. Acetylation of sulfanilamide has been investigated in slices of guinea-pig liver by Klein and Harris (1) and in homogenate and extracts of pigeon liver by Lipmann (2). In these investigations the aromatic amines, sulfanilamide and p-aminobenzoic acid, were used as acetyl acceptors because conjugation could be followed conveniently by determination of the disappearance of diazotizable amine. That acetyl sulfanilamide was the product formed was established by isolation. In these systems acetate was the most potent acetyl donor, while acetoacetate and pyruvate showed a lesser effect. The results suggest that the immediate reactant is either acetic acid itself or a C_2 compound which under these conditions arises more readily from acetate than from pyruvate or acetoacetate. Five times as much acetylsulfanilamide was formed under aerobic than under anaerobic conditions (2). The linking of acetylation with an oxidative process is further illustrated by the finding that the acetate effect is abolished by oxidation poisons such as cyanide or iodoacetate (1). Although oxygen does not enter into the formal equation of the acetylation by acetate, aerobiosis is required to supply the energy for the condensation. Lipmann has demonstrated that acetylation of sulfanilamide takes place also anaerobically if "energy rich" phosphate in the form of adenosine triphosphate is provided (2). It is not likely that the function of adenosine triphosphate consists in the formation of acetylphosphate since the addition of this compound does not elevate the level of anaerobic sulfanilamide acetylation. The enzyme system concerned in the acetylation of these aromatic amines is most abundant in pigeon liver and has been found to be extractable (2).

A different enzyme seems to be responsible for the acetylation of α amino acids by acetate. Bloch and Borek (31) found, with the aid of deuterio acetate, acetylation not only of l-phenylaminobutyric acid but also of the natural amino acids, leucine and phenylalanine. The yields of acetylamino acids were much higher with slices from rat liver than from those of guinea pig or pigeon. After incubation with labeled acetate, amino acid and non-isotopic acetylamino acid as a carrier, the quantities of acetylamino acid formed could be calculated from

the isotope content of the isolated acetylamino acid. The amounts of acetyl compound obtained with phenylaminobutyric acid were much greater than with the two natural amino acids, presumably because the acetyl derivative of the foreign amino acid is not further metabolized while the acetylated natural amino acids seem to be short-lived intermediates. Thus, under the same experimental conditions the acetylderivative of a natural amino acid, acetylglycine, was found to be deacetylated.

These results indicate that aromatic amines and both natural and foreign α-amino acids effectively utilize acetic acid as a source of acetyl groups. It is evident that whatever the nature of the C_2 compound which is the immediate acetyl precursor, it can readily be formed from acetic acid itself. The effect of adenosine triphosphate on the acetylation reaction observed by Lipmann (2) suggests that the condensation between the carboxyl group of acetic acid and the amine involves the elimination of phosphoric acid rather than of water. Since the effect of adenosine triphosphate cannot be attributed to the intermediary formation of acetylphosphate, the possibility remains to be explored that phosphorylated sulfanilamide or an acetyl derivative of adenosine triphosphate may be formed as intermediates.

The existence of a second mechanism of acetylation and of another source of acetyl groups is indicated by results obtained with labeled alanine (29, 30). Alanine which contains deuterium at the α and β carbon atoms causes the excretion of deuterio acetyl groups when phenylamino butyric acid is simultaneously administered, but only a slight excess of isotope is found in the acetylation product when sulfanilamide or p-aminobenzoic acid are fed as acetyl acceptors. Since alanine, in contrast to acetate, provides acetyl groups for α amino acids ony, its effect cannot be explained on the basis of intermediate formation of acetic acid. The effect of alanine may reasonably be ascribed to the condensation of a C_3 compound, presumably pyruvic acid, with an imino acid and subsequent decarboxylation of the condensation product to an N-acetyl derivative. In this case the failure of alanine to acetylate aromatic amines can be readily understood since imino acids cannot arise from aromatic amines.

The above result which provides experimental evidence for the acetylation schemes suggested by Knoop (8) and du Vigneaud et al. (20) is of interest in several respects. The reaction:

$$CH_3COCOOH + RCH(NH_2)COOH + \tfrac{1}{2}O_2 \rightarrow RCH(NHCOCH_3)COOH + CO_2 + H_2O \quad [1]$$

can be assumed to proceed with an overall decrease of free energy so that it becomes unnecessary to postulate the intermediate formation of an active C_2 compound as the immediate precursor of N-acetyl groups. This is in contrast to the acetylation of amines by acetic acid which is an endergonic process and therefore requires coupling with an energy yielding reaction. The conversion of pyruvic acid to acetyl amino acid, by effecting the degradation of a three carbon compound to a C_2 unit without intermediate formation of free acetic acid, offers a possible mechanism for the conversion of pyruvate to the oxidation level of

acetate, a step in the oxidation of carbohydrate which is little understood at present.

That there may exist a connection between acetylation reaction [1] and the formation of acetoacetate from pyruvate has been pointed out by Krebs (32). The fact that ketone body formation from pyruvate in liver is greatly enhanced in the presence of ammonia (33, 34) can be explained on the basis of acetylamino acid formation from keto acid, ammonia and pyruvate, and subsequent condensation of acetyl groups to acetoacetate. Formation of an N-acetyl derivative from pyruvate has so far been demonstrated only with phenylamino-butyric acid but not with natural amino acids. Also, the ability of N-acetyl groups to yield acetoacetate remains to be tested.

The direct participation of acetoacetate as an intermediate in the acetylation of amines by acetic acid has been considered but appears unlikely in view of the following findings (29, 30): deuterioacetyl groups are formed from butyric acid labeled with heavy hydrogen either at the α or the γ carbon atoms, indicating that both two carbon moieties of the intermediate acetoacetic acid are employed for acetylation. Secondly, deuterioacetic acid which also contains C^{13} at the carboxyl position affords N-acetyl groups in which the relative proportion of C^{13} and D remains unchanged. The formation of acetoacetate from such doubly labeled acetate would entail replacement of deuterium by normal hydrogen, but no loss of C^{13}, as the result of the following reactions:

$$2CD_3C^{13}OOH \underset{+H_2O}{\overset{-HDO}{\rightleftharpoons}}$$

$$CD_3C^{13}OCD_2C^{13}OOH \underset{+H}{\overset{-D}{\rightleftharpoons}} CD_3C^{13}ONa{=}CDC^{13}OOH \quad [2]$$

$$\downarrow$$

$$(CD_3C^{13}O) + (CH_3C^{13}O)$$

Any acetyl formed by way of acetoacetate should therefore have a diminished $D:C^{13}$ ratio. Since the conversion of acetate to N-acetyl is not accompanied by any loss of carbon-bound deuterium, acetoacetate may be ruled out as an intermediate step. The fact that the $D:C^{13}$ ratio is not affected in a reaction in which acetic acid is utilized also speaks against a rapidly occurring interconversion of acetate and acetoacetate in the intact animal. The significance of this finding with regard to fatty acid catabolism will be discussed later.

Metabolism of acetylamino acids. The occurrence of a process by which acetyl groups can be distributed between amino acids is suggested from data obtained with labeled acetylamino acids (35). These experiments were carried out in order to determine whether the acetyl derivatives of natural amino acids can be biologically deacetylated. Any acetic acid liberated from these compounds should become demonstrable by its capacity to acetylate foreign amines. When the deuterio acetylderivatives of glycine, alanine, leucine or glutamic acid were fed together with phenylaminobutyric acid, deuterium was found in the excreted

acetyl phenylaminobutyric acid, but the level of isotope was very much higher than that obtained after feeding of equivalent quantities of labeled acetic acid. This result was interpreted as showing that the foreign amino acid had received acetyl by direct transfer from the administered acetylamino acid and that in the process no intermediate formation of free acetic acid had occurred. The high isotope concentrations in excreted acetylamine were not obtained when p-amino benzoic acid was the foreign amine. In the transfer of acetyl groups an α amino acid is therefore necessary as an acetyl acceptor. Only the acetyl derivatives of natural amino acids can serve as acetyl donors; the labeled acetyl derivatives of d-alanine, d-leucine and sarcosine failed to yield deuterio acetyl groups. These results paralled those obtained with acetyl d-amino acids in growth experiments and substantiate the conclusion that no biological mechanism is available for the deacylation of acetyl d-amino acids. The fact that α-amino acids are necessary both as acetyl acceptors and as acetyl donors may perhaps indicate that the transfer reaction is coupled with dehydrogenation at the α carbon atom of the amino acids and that in order to allow the reaction to occur the steric configuration of the hydrogen at the α carbon must be that of the natural amino acids. Data on the acetyl transfer reaction have so far been obtained only with phenylaminobutyric acid as acetyl acceptor. *In vivo* the acetyl derivatives of natural amino acids do not accumulate sufficiently to permit their isolation. Transacetylation as a process which operates between two natural amino acids therefore remains to be demonstrated.

Organic chemical reactions representing intermolecular transfer of acetyl groups to amino acids have been known from the investigations of Bergmann et al. Bergmann, du Vigneaud and Zervas (36) found that the acetyl groups of diacetyl diketopiperazine are readily shifted to free amino acids. The reaction proceeds in aqueous solution at room temperature:

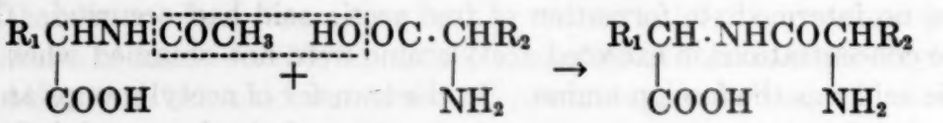
Acetyl anhydropeptide + amino acid → anhydropeptide + acetylamino acid [3]

Acetyl transfer was also shown to occur with a histidine derivative which contained the acetyl substituent at one of the imidazole nitrogen atoms (37). Evidently an acetyl group is more reactive when linked to a secondary nitrogen and its transfer to a primary amino group *in vitro* will therefore occur spontaneously. This raises the possibility that the acetyl donor concerned in the biological transfer of acetyl groups may be an N-acetylpeptide rather than a free acetylamino acid. On the basis of these model reactions Bergmann and Zervas (37) suggested that acetylamino acids might play a rôle in the biological formation of peptide bonds. This hypothesis assumes added significance in view of the demonstration that the acetyl derivatives of natural amino acids are biologically formed (31) and that a mechanism exists for the shifting of acetyl groups from one amino acid to another (35).

The formation of an acetylamino acid presumably requires a very similar quantity of energy as the formation of a peptide bond, i.e., 2.5 to 3.0 K cal. (38). This energy can readily be supplied by the acetylation mechanism [1] which involves the conversion of pyruvic acid to N-acetyl. On the other hand, it is

not clear which energy yielding step facilitates the acetylation of amino acids by acetate.

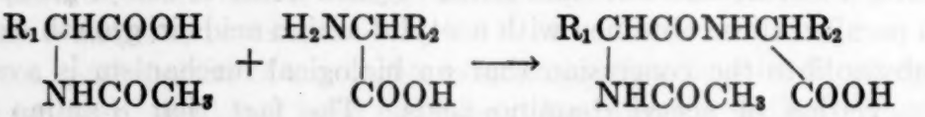
Acetyl amino acids may react with free amino acids in two ways: 1, by acetolysis:



[4]

This reaction should involve only a small change of free energy.

Or 2,



[5]

The second reaction, which would yield an acetylpeptide, is analogous to the acetylation of amino acids by acetic acid, an acetamino acid being the acetylating agent instead of acetic acid. It may be pointed out that as a result of acetylation, the amphoteric amino acids are converted into strong acids (39).

The ability of the animal organism to utilize acetylamino acids is also illustrated by the finding that, with the exception of α-N-acetyl lysine (40), the acetyl derivatives of essential amino acids are nutritionally equivalent to the free amino acids. This has been tested in growth experiments with the acetyl derivatives of tryptophane (41, 42), phenylalanine (43), and histidine (44). The ability of acetylglycine to raise the level of hippuric acid excretion in rabbits above the normal suggests conversion of the acetylderivative to the free amino acid (45). In those instances in which the acetyl amino acid can take the place of an indispensable amino acid, the corresponding α keto or α hydroxy acids will likewise be capable of supporting growth (46). It is therefore not clear whether regeneration of the free amino acid from the acetyl derivative involves direct deacetylation by hydrolysis or whether it is due to a process which regenerates the amino acid by way of the α-keto or α-hydroxy acid. The observation that α-N-acetyllysine cannot support the growth of rats on a lysine deficient diet has been interpreted by Neuberger and Sanger (40) as suggesting rapid oxidation of the ϵ amino group of lysine prior to deacetylation. However, it is also possible that the α-keto acid is formed directly without passing through free lysine as an intermediate. The α-keto acid of lysine is not known, but it may be presumed to be incapable of substituting for lysine in supporting growth because the α-nitrogen of lysine, in contrast to that of other amino acids, cannot be replaced metabolically by nitrogen from other sources (47) and because d-lysine cannot support growth (48). On the other hand, Neuberger and Sanger (40) found ϵ-N-acetyllysine to be nutritionally equivalent to lysine, suggesting a process of metabolism in which the acetyl group is removed by hydrolysis to regenerate free lysine. In agreement with this observation Bloch and Rittenberg found deuterio ϵ-N-acetyllysine to yield acetyl groups for the acetylation of foreign amines with an isotope content to be expected from hydrolysis of the acetyl amino acid (unpublished experiments).

The enzyme systems catalyzing deacetylations show specificity with respect to the spatial configuration of the acetyl amino acids. Thus the acetyl derivatives of d-tryptophane (42, 49), d-phenyl alanine (17) and d-leucine (35) are metabolically inert; acetyl d-phenylalanine has been recovered unchanged from the urine of the experimental animals (17). The sulfur of acetyl d-cystine is resistant to oxidation while acetyl l-cystine is readily metabolized (50). The unavailability of acetyl d-amino acids must be due to the inability of animal tissues to deacetylate because the corresponding free d-amino acids can be utilized and can replace nutritionally their optical antipodes (49, 51, 52). This is further illustrated by the failure of the deuterio acetyl derivatives of d-alanine and d-leucine to yield labeled acetyl for the acetylation of foreign amines (35). Some significance may be attached to the fact that whereas deamination occurs readily with both stereoisomers of most amino acids, only the acetyl amino acids of natural configuration are metabolized by animal tissues.

Acetylation may be associated with the formation of amino acids from keto acids and ammonia but it can also occur subsequent to amino acid synthesis. It is thus conceivable that acetylation may be concerned in the catabolism of amino acids. The following findings are of interest in this connection. The observation already mentioned of Neuberger and Sanger (40) that α-N-acetyl-lysine cannot replace lysine for support of growth indicates that the compound is not deacetylated but that it may be directly converted to the nutritionally ineffective α keto acid. Secondly, it has been found that on feeding of acetyl-l-phenylalanine to rats, phenylacetic acid can be isolated from the urine in the form of phenaceturic acid. (Unpublished observation of the author.) Since this compound was not obtainable after the feeding of free l-phenylalanine it would appear that the acetyl derivative was deaminated more readily than the free amino acid.

Formation of acetic acid. The first suggestions of a formation of acetic acid in quantity in animal metabolism were contained in experiments of Knoop (53) and Dakin (54) on the biological degradation of phenyl-substituted fatty acids. The excretion of either phenylacetic acid or benzoic acids (or their conjugation products with glycine), depending on the number of carbon atoms in the aliphatic chain of the test substance, was most reasonably explained as resulting from the successive removals of two carbon fragments, by hydrolysis of an intermediate β keto acid. The exact nature of the two carbon fragments could not be ascertained, but chemical considerations pointed to acetic acid or an acetyl compound. Acetic acid has been proposed repeatedly as an intermediate in the main path of carbohydrate oxidation. Thunberg (55) suggested that oxidative decarboxylation of pyruvic acid gave rise to acetic acid, two molecules of which were dehydrogenated to form succinic acid. In the course of their metabolism, several amino acids form intermediates which are identical with products of either carbohydrate or fatty acid breakdown, pointing to protein as a potential source of acetic acid. Thus it was conceivable that the metabolic paths of the three major dietary and tissue components, fat, carbohydrate and protein, would converge at the two carbon stage and that, once this

stage had been reached, only one common mechanism of oxidation would be required. It is interesting to note that the principal aspects of this theory of intermediary metabolism which, at the time, rested on little experimental evidence, have required revision in a few details only. Experimental evidence in its favor continues to accumulate.

The occurrence of acetic acid in animal tissues. As an intermediate in the main chain of metabolic reactions, acetic acid should continually arise in large quantities. Analysis of tissues and body fluids reveals that in the equilibrium state, acetic acid is present in quantities which are barely detectable (56). Acetic acid in the form of its dinitrophenylhydrazide has been isolated from large quantities of beef liver (57), but it is not clear whether the material thus obtained was an artefact formed during the isolation procedure. Specific methods for the determination of small quantities of acetic acid in biological mixtures are not available; it may also escape detection because it seems to be firmly bound to protein in biological media (58, 59). Nevertheless, even if acetic acid were found to be practically absent, the suggested rôle of acetic acid as a major product of catabolism would not be contradicted. The condition which has to be satisfied, and which applies to all metabolites which lie on the main metabolic paths, is a capacity of the tissue to metabolize the intermediate as rapidly as it is formed. The concentration of the metabolite at any one time will then be irrelevant. The insignificant concentration of acetic acid and other fatty acids of intermediate size in animal tissues has been cited as evidence against the theory of β oxidation (60). If this objection were valid, the same reasoning would apply to numerous commonly accepted intermediates of protein and carbohydrate metabolism, because the demonstration of their presence in normal tissues has not been experimentally feasible.

Evidence for the occurrence of a metabolite which does not normally accumulate in sufficient amounts to permit analytical detection can be obtained with the aid of the isotope dilution method. If the isotopic analogue of a suspected intermediate is administered to an animal, or added to an isolated system, and subsequently recovered either as such or in combined form, a diminished isotope content of the isolated material will indicate that the test substance had merged with identical unlabeled molecules formed in the tissues. Bernhard, in an investigation of the acetylation of foreign amines with labeled test substances (21) found that the acetyl groups of excreted acetyl sulfanilamide contained 5 per cent to 20 per cent as much isotope as the deuterio acetic acid added to the diet. He concluded that the fraction of acetyl groups contributed by exogenous acetate was related to the difference of isotope content in the test substance and excreted acetyl. These findings suggested that the acetyl precursor consisted of a mixture of dietary and endogenous acetic acid. The possibility that the acetic acid excreted by the animal in the form of acetylamine was a representative sample of the acetate existing in the metabolic pool offered an experimental approach to a quantitative determination of this metabolite in the intact animal. From such experiments, Bloch and Rittenberg (30) have concluded that the isotope dilution obtaining in the acetylation process can be attributed to the merging of labeled

dietary acetate with acetate arising in metabolism. The magnitude of the dilution factor indicates that the quantities of acetic acid produced are large.

In order to ascertain the correctness of this conclusion it was necessary to consider the following sources of isotope dilution apart from that caused by endogenous acetate: 1, rate of the acetylation reaction; 2, labilization of the C—H bond in the methyl group of deuterio acetic acid; 3, acetylation by precursors other than acetic acid.

1. Since the acetyl derivatives of foreign amines are not stored by the animal but excreted into the urine soon after their formation, the rate of the reaction need not be considered. The isotope concentration of excreted acetyl is the same irrespective of the length of time during which labeled acetate is administered. 2. In one of the acetylation experiments, a preparation of acetic acid which contained deuterum in the methyl group and a carboxyl group labeled by C^{13} was administered simultaneously with phenylaminobutyric acid. The acetyl group of the excreted acylamino acid contained the two isotopic markers in much smaller concentrations, but the ratio of C^{13}:D had remained unchanged. This finding eliminates loss of deuterium as a source of isotope dilution and also attests to the biological stability of the C—H bonds in the methyl group of acetic acid. 3. If the acetyl groups were derived not only from acetic acid but also from other precursors, such acetyl groups would be non-isotopic and hence dilute the deuterio acetyl groups which originate from labeled acetate. Evidence for the contention that acetate may be the only source of acetyl groups for aromatic amines (sulfanilamide, p-aminobenzoic acid) and the major one in the acetylation of α amino acids has been discussed above. It is based on the finding that in identical feeding experiments, acetic acid effects much higher isotope concentrations in excreted acetyl than any other compound tested. According to Bernhard (27), labeled ethanol is a better source of acetyl than acetic acid, but in the experiments of Bloch and Rittenberg (29) ethanol and acetate were equally effective. On the basis of available evidence it seems likely that alcohol is rapidly oxidized to acetic acid and thus becomes a source of acetyl groups. Still, the possibility exists that a C_2 compound which is readily available from both alcohol and acetate is the immediate acetylating agent.

The finding that alanine is a source of acetyl groups for foreign α amino acids but not for foreign aromatic amines has been attributed to a condensation of pyruvic acid with the imino acid derived from phenylaminobutyric acid. While these results establish the existence of a second source of acetyl groups for α amino acids, they do not invalidate the contention that in the acetylation of aromatic amines acetic acid serves as the sole source of acetyl groups. It was therefore reasonably well established that in feeding experiments with labeled acetate the change in isotope concentration occurring in the acetylation reaction provides a direct measure of endogenous acetate production. By applying the equation for isotope dilution (61), the quantity of acetate formed by the rat in a 24-hour period was calculated to be 15 to 20 mM per 100 grams, or approximately one per cent of the body weight. This value could be in error in either direction if the ingested acetic acid failed to mix adequately with the acetate

of the metabolic pool, in which case either the dietary material or the endogenous acetate might be employed preferentially for acetylation. However, the following considerations lend support to the correctness of the above calculation: the isotope concentration of excreted acetyl was found to be directly proportional to the total quantity of labeled acetate administered per unit of body weight, and hence the calculated value for acetate production in vivo is independent of dosage variation over a wide range. This finding points to a rapid merging of dietary acetate and the acetic acid formed in the tissues.

In vitro studies by Klein and Harris (1) indicate that the acetylation reaction is confined to the liver. The sample of acetate which becomes trapped by forming acetylamine is therefore representative only of the hepatic acetate pool. Of the acetic acid of extrahepatic origin, only the fraction which escapes oxidation at the site of formation and is dispatched to the liver will be measured in the acetylation reaction. The resultant error cannot be assessed at the moment, because it is not known to what extent acetyl-yielding reactions take place in extrahepatic tissues.

Lorber et al. (62) perfused the isolated heart with labeled acetic acid and found that the latter was apparently diluted by normal acetate in amounts corresponding to those calculated for the intact rat (30).

R. Q. measurements of animals in the fasting state (63) and after hepatectomy (64) have been interpreted as indicating fatty acid oxidation in organs other than the liver. Stadie (65) has calculated that in the animal depleted of carbohydrate stores, the energy requirements cannot be met by hepatic production of ketone bodies. The occurrence of extrahepatic fat oxidation is illustrated by experiments of Lehninger, who showed that palmitic and octanoic acids are metabolized by a heart muscle preparation to yield succinate and ketoglutarate (66). An independent estimate of the size of the metabolic acetate pool has been made on the assumption that a molecule of fatty acid of average size (C_{16}) yields on degradation eight molecules of acetate. With the aid of available data on rates of fatty acid regeneration (67) it is possible to calculate how much acetate should arise in a given period of time if fatty acids were the only source. The quantity thus calculated agrees closely with that obtained experimentally by isotope dilution measurements.

Acetic acid formation from fatty acids. Fatty acid oxidation[1] has been investigated in a variety of systems, viz., in the fasting or diabetic animal, in perfused livers, in various liver preparations, and in the intact animal with the aid of labeled test substances. Under all conditions except those mentioned last, acetoacetic acid is the principal product of the oxidation process. Leloir and Muñoz (68) detected acetic acid in the course of octanoate oxidation by liver, but the amount was insignificant compared with the quantities of ketone bodies which accumulated. Deuel and co-workers (69, 70) found a uniform increase of ketone body excretion with increasing chain length of the fatty acid, administered as salt or ethyl ester, to starving rats. In isolated liver, the yields of

[1] For a detailed consideration of this subject the reader is referred to the review by Stadie (60).

ketone bodies per molecule of fatty acid obtained by different investigators differ widely (71 to 74), depending apparently on the previous treatment of the liver tissue. *In vitro*, fatty acids appear to become increasingly resistant to oxidation as the length of the chain increases. The depressing effect of higher fatty acids on the respiration of isolated tissue, noted by Quastel and Wheatley (71), has been attributed to their surface activity. Lehninger has been able to show, however, that oxygen uptake can be restored and that the higher fatty acids can be oxidized by addition of adenosine triphosphate to the liver system (74). Under the experimental conditions of this investigator the carbon atoms of some even and odd numbered fatty acids (C_8, C_7) could be accounted for as acetoacetate in nearly quantitative yields.

Since acetoacetic acid accumulates as the principal product of fatty acid oxidation, it is not surprising that acetoacetate rather than acetate has frequently been emphasized as the primary oxidation product. On the other hand, recent experiments have furnished clear evidence for the correctness of the principle of β oxidation as proposed by Knoop (53). Stepwise degradation of a biologically occurring fatty acid by elimination of two carbon atoms was first demonstrated by Schoenheimer and Rittenberg (75). Palmitic acid isolated from the tissue fat of animals which had received deuterio stearic acid had a sufficiently high isotope content to show that it arose directly from the C_{18} acid by shortening of the chain. Their data indicated that myristic acid had been similarly formed by subsequent elimination of two carbon atoms from palmitic acid. The further degradation could not be studied because tissue fat contains only traces of the acids with less than 14 carbon atoms.

The two carbon fragment which is detached from the fatty acid chain has not been identified as such, although its intermediate formation has been clearly established. Weinhouse, Medes and Floyd (76) have investigated the oxidation of octanoic acid which was labeled at the carboxyl group with C^{13}, in liver slices. The acetoacetic acid which accumulated as the main product, contained labeled carbon in nearly equal concentrations at the carbonyl (β carbon) and carboxyl positions. Acetoacetate with such isotope distribution could not have resulted from the splitting of the C_8 acid into two/four carbon compounds (multiple alternate oxidation), nor could it have been formed exclusively from carbon atoms 5 to 8 of the octanoic acid (classical β oxidation). Acetoacetate must have been formed, at least in part, by random condensation of C_2 fragments.

The ability of fatty acids to yield acetyl groups in the intact animal can be tested by exploiting the fact that foreign amines are converted in vivo into metabolically inert acetyl derivatives. Since acetic acid is an effective source of such acetyl groups, the assumption can be made that the appearance of labeled acetyl amine after feeding of labeled precursor is a measure of acetyl formation. The following deuterio-fatty acids were found by Bloch and Rittenberg (29) to be sources of deuterioacetyl groups: butyric acid, n-valeric acid, isovaleric acid and myristic acid; propionic and undecylic acids failed to yield acetyl. The results obtained were found to conform with the assumption that β oxidation and removal of acetyl groups from the β keto acids was the principal event in the

metabolism of the fatty acids. It is clear that deuterium is not always an adequate label for the study of carbon chain interconversions, and the results obtained on acetyl formation are in most cases qualitative only. All deuterium attached to the β carbon atoms will be lost by oxidation and further loss of deuterium is to be expected due to keto-enol tautomerism involving the methylene hydrogens adjacent to the keto groups. Thus, α, β dideuteriobutyric acid yields acetyl of much lower isotope concentration than butyrate which contains deuterium at the β and γ positions. If acetoacetate is the intermediate in the butyrate-acetyl conversion, loss of deuterium from the labile α position and therefore a lowered isotope content of the acetyl fragment derived from the carboxyl and α carbon atom of butyric acid would result. The behavior of the two preparations of deuterio butyric acid suggests breakdown of the intermediary β keto acid into two identical acetyl fragments.

The various β keto acids which are formed successively from the higher fatty acids will suffer the same loss of deuterium at the α carbon as the α fragment of butyric acid, so that only the deuterium bound to the terminal carbon remains in stable linkage. In the intact animal, the long chain fatty acids, in contrast to those having a small number of carbon atoms, are only partly catabolized immediately to yield acetyl groups. For example, a large portion of administered myristic acid will be deposited in the tissues as such and will also be converted to palmitic and stearic acids by chain elongation. In this case the deuterium content of excreted acetyl will not be a quantitative measure of the amounts of acetyl produced.

Metabolism of odd numbered fatty acids. The odd numbered straight chain fatty acids are not encountered in animal metabolism but they are oxidized in vivo and in vitro as readily as the corresponding even numbered fatty acids. In isolated liver (72) and in the intact animal (77) the fatty acids possessing an odd number of carbon atoms (except propionic acid) are precursors of ketone bodies although the quantities produced are smaller than those afforded by the neighboring fatty acids with an even number of carbon atoms. Propionic acid is presumably oxidized to a three carbon compound which can be converted quantitatively to carbohydrate. Bloch and Rittenberg have concluded that pyruvic acid cannot be an intermediate in this conversion since deuteriopropionic acid, in contrast to labeled alanine, failed to provide acetyl groups in the acetylation of phenylaminobutyric acid (29). Deuterio n-valeric acid formed labeled acetyl to an extent which indicated that one two-carbon fragment arose per mole of valerate. Cohen found that isovaleric acid is a much more potent source of acetoacetate in liver slices than n-valerate (73). In the intact animal labeled isovalerate yielded more deuterioacetyl than equivalent amounts of the n-valerate (78). From the data obtained, it could not be decided whether the breakdown of this branched chain acid proceeds by way of demethylation to butyric acid or by initial oxidation at the tertiary carbon atom to acetone and a C_2 fragment. Undecylic acid labeled with deuterium at the 10 and 11 positions did not yield deuterio acetyl. β oxidation of this eleven carbon acid may be visualized to lead eventually to a three carbon fragment which contains the

isotopic hydrogen but which, like propionic acid, would not be degraded directly to a C_2 unit. The behavior of the odd numbered fatty acids can be readily understood if it is assumed that their metabolism proceeds in a fashion analogous to that of the even-numbered fatty acids with the difference that their terminal three carbon atoms are, like propionic acid, converted to carbohydrate. From an odd numbered acid having n carbon atoms, only n−3 carbon atoms will then be available for ketone body formation so that less acetoacetate will be formed per molecule than from the neighboring even numbered fatty acid.

The demonstration of the ketogenic effect of n-valeric acid (72, 77) has had an important bearing on the development of more recent theories of fat oxidation. This finding could not be adequately explained by either classical β oxidation or by the hypothesis of multiple alternate oxidation. MacKay et al. (77) suggested that the five carbon chain of valerate was degraded to a three carbon and a two carbon unit and that two of the latter combined to yield acetoacetate. It became evident therefore that ketone bodies could be formed by recondensation of two carbon fragments split off from the fatty acid chain as well as by direct oxidation.

Formation of acetyl from amino acids. It is generally believed that α amino acids are catabolized by oxidative deamination to α keto acids and subsequent oxidative decarboxylation to acids containing one carbon atom less. In this case amino acids which yield saturated fatty acids as intermediates should be sources of acetyl groups. This possibility has been studied with the amino acids leucine and valine which contained carbon chains labeled by deuterium (78). Deuterioleucine yielded labeled acetyl groups to the same extent as isovaleric acid indicating that this acid was formed as an intermediate. The ketogenic effect of leucine (73, 79) may be explained on the same basis. Valine and isobutyric acid both are nonketogenic and are also ineffective as sources of acetyl groups. No information is available concerning the steps by which isobutyric acid is further metabolized except that three of its four carbon atoms are convertible to glycogen (80). It should be of interest to ascertain whether the ketogenic action of phenylalanine and tyrosine is associated with acetyl formation, particularly whether the ring structure of these amino acids can be degraded to two carbon units.

Mechanisms of fatty acid oxidation. A consideration of all pertinent data obtained in experiments with intact animals and with *in vitro* systems leads to the conclusion that successive β oxidation, followed by removal of C_2 fragments, is the principal event in fatty acid oxidation. For a number of years undue emphasis was placed on experimental observations which appeared to contradict β oxidation as originally proposed by Knoop. It should be pointed out that the data which led to a criticism of the theory of β oxidation were obtained with either isolated tissues or starving or diabetic animals. Since under these conditions acetoacetic acid and not acetic acid accumulates, the ketone bodies came to be regarded as primary oxidation products not only of the terminal four carbon atoms but of the entire fatty acid chain. Moreover, the theory of multiple alternate oxidation was advanced as a mechanism for fat oxidation

because, *in vivo* and *in vitro*, the fatty acids containing six and more carbon atoms gave a higher yield of acetoacetic acid than butyric acid, while Knoop's theory provided for the formation of only one mole of keto acid regardless of the length of the chain. However, the formation of ketone bodies in excess of one mole per mole of fatty acid is not at variance with β oxidation if it is ascribed to the recondensation of acetic acid to acetoacetic acid. The occurrence of this reaction was early demonstrated by Loeb (3) and Friedemann (81), who observed acetoacetate formation on perfusing liver with acetate. This finding has been confirmed repeatedly and under a variety of conditions, viz., by Jowett and Quastel and by Leloir and Muñoz in liver slices (72, 68), by Monguio and by MacKay et al. in intact animals (4, 5), and with C^{13} labeled acetate in starving animals by Swendseid et al. (82) and by Medes et al. in liver slices (83). It was recognized by MacKay et al. (5) that the synthetic formation of acetoacetate from acetate provided a basis for a theory of fat oxidation which retained Knoop's basic concept of β oxidation and ascribed the formation of "excess" acetoacetate to a subsequent event, namely, a recombination of two molecules of acetate. The data of Weinhouse et al. with carboxyl labeled octanoic acid (76) demonstrate that acetoacetate can be formed by random coupling of C_2 fragments which are split off from the fatty acid chain. There are, however, two possible pathways which conform with the observed isotope distribution:

$$\text{(a)}\ CH_3(CH_2)_6\,C^{13}OOH \rightarrow 4C_2 \rightarrow 2CH_3C^{13}OCH_2C^{13}OOH \qquad [6]$$

$$\text{(b)}\ CH_3(CH_2)_6\,C^{13}OOH \rightarrow C_2^{13} + C_2 + CH_3CH_2CH_2COOH$$

$$CH_3CH_2CH_2COOH \rightarrow CH_3COCH_2COOH \qquad [7]$$

$$C_2^{13} + C_2^{13} \rightarrow CH_3C^{13}OCH_2C^{13}OOH$$

Reaction (a) does not indicate the mechanism which yields the four C_2 fragments from the fatty acid chain; it implies that all of the acetoacetate is of synthetic origin. Reaction (b) is a modified version of classical β oxidation and retains the view that the four terminal carbon atoms are a direct source of acetoacetic acid. In this case the accumulated acetoacetic acid would be a mixture of "primary" and synthetic molecules. In similar experiments with carboxyl labeled butyric acid the resulting acetoacetate contained an unequal distribution of C^{13} at the carboxyl and carbonyl carbon atoms (85). This acetoacetate could therefore not have been formed by random coupling of two equivalent C_2 fragments. Medes et al. (85) have pointed out that their data can be represented by the following equation:

$$CH_3CH_2CH_2COOH \rightarrow CH_3COCH_2COOH \rightleftarrows 2CH_3COOH \qquad [8]$$

In this case rapid interconversion of acetate and acetoacetate has to be postulated. This is true for the synthesis of acetoacetate in liver (84) and kidney (86). The reverse reaction was found by Lehninger (59) to proceed slowly in muscle mince, but it is not clear whether in liver and kidney acetoacetate is split at a sufficient rate to account for the redistribution of isotope in accordance with the above equation.

A similar explanation may apply to the results obtained by Morehouse (87) and by Morehouse and Deuel (88), who fed two preparations of deuterio butyric acid and deuterio caproic acid to starving rats and isolated β-hydroxybutyric acid from the urine. The excreted hydroxybutyrate contained nearly twice as much deuterium when β-γ-dideuteriocaproate was fed as the acid excreted after administration of caproate labeled at the α and β positions. It is evident that a splitting of the hexanoic acid into three two carbon units followed by recondensation to acetoacetic acid should yield hydroxybutyric acid containing the same isotope concentration in both cases. On the other hand, if the excreted β-hydroxybutyric acid was derived mainly from "primary" acetoacetate representing the intact chain of carbon atoms 3 to 6 of caproate, and was formed only in part by recondensation of C_2 units, then β-γ-dideuteriocaproate should yield a hydroxybutyric acid of higher deuterium concentration than α-β-dideuteriocaproate.

Stadie (60) has pointed out that the data obtained by Weinhouse et al. with labeled octanoate are not inconsistent with the theory of multiple alternate oxidation if the random distribution of labeled carbon is the result of a splitting and reformation of acetoacetate. Since it is agreed that the formation of a C_2 fragment is an obligatory step in the breakdown of fatty acids, it remains to be decided whether acetoacetate is the primary oxidation product or whether its formation represents an alternative path for the disposal of C_2 fragments which are initially formed.

Some indications as to the rôle of acetoacetate in fatty acid oxidation are contained in experiments on the acetylation of foreign amines by acetic acid (30). It has been pointed out that the results from these experiments eliminate acetoacetic acid as an intermediate in the conversion of acetate to N-acetyl groups. From the same analytical data it can be concluded[2] that in the intact animal a rapid interconversion of acetate and acetoacetate is unlikely to occur. Doubly labeled acetic acid ($CD_3C^{13}OOH$), when fed and recovered as acetyl phenylaminobutyric acid, retains deuterium and C^{13} in identical relative concentrations. Had acetate been converted to acetoacetate and regenerated, replacement of deuterium by normal hydrogen and loss of deuterium by enolization should have altered the $D:C^{13}$ ratio. The accumulation of acetoacetic acid in fatty acid oxidation in vitro, or in the urine of animals in ketosis may therefore be ascribed to circumstances which interfere with the normal disposal of acetyl or acetate. The isolated liver or liver preparations as ordinarily employed contain little available carbohydrate, and therefore lack the requisite dicarboxylic acids for the oxidation of the intermediates of fatty acid breakdown. Liver tissue appears to have only a limited capacity to oxidize fat beyond the C_2 stage but dispatches the products to extrahepatic tissues for complete combustion. In the isolated liver acetyl will therefore accumulate and become stabilized in the form of acetoacetate. This point is clearly illustrated by the experiments of Lehninger (89) who found that in preparations of washed liver cells, acetoacetate is the

[2] The author is indebted to Prof. D. L. Thomson for having called attention to these implications contained in the experimental data.

sole product of octanoate oxidation, but that on addition of fumarate the quantities of acetoacetate are diminished in favor of α-ketoglutarate, succinate, and citrate. It becomes unnecessary then to assume that acetoacetate lies on the main path of fat oxidation. Acetoacetic acid is utilized under a variety of conditions, but this is probably preceded by its splitting into two carbon units.

Formation of acetyl from pyruvate. There exists at present no conclusive evidence to indicate whether pyruvic acid enters the citric acid cycle by combining directly with oxaloacetic acid or whether this condensation is preceded by a conversion of pyruvate to an intermediate which has the oxidation level of acetic acid. Formation of acetate from pyruvate can be demonstrated to occur in animal tissues, either aerobically (90, 91, 92) or anaerobically by dismutation (90, 91, 93) but this reaction is not believed to represent an important pathway, because under the same conditions acetate itself is oxidized relatively slowly. It is considered more likely that the oxidation product of pyruvate is represented by a two carbon compound which is metabolically more reactive than acetic acid itself. A phosphoroclastic splitting of pyruvate into acetyl-phosphate and formate (or $CO_2 + H_2$) as it occurs in bacteria (94) has not been observed in animal tissues.

According to Barron et al. (95), acetate can be shown to accumulate when liver or kidney slices oxidize pyruvate in the presence of fluoroacetate. According to these investigators, this compound seems to inhibit the oxidative disappearance of acetic acid by competing with acetate for the enzyme. The accumulation of acetate indicates that fluoroacetate may exert its effect by blocking condensations involving the reactive acetyl compound, which may then become stabilized in the form of acetate.

In the presence of malonate, pyruvate oxidation by liver slices or mince affords acetoacetate in quantities up to 25 per cent of the pyruvate metabolized (33, 34, 96). Pyruvate is quantitatively converted to acetoacetate by the washed liver cell preparation of Lehninger (66). When fumarate was added to the same system, acetoacetate formation was suppressed in favor of an increased yield of di- and tri-carboxylic acids. The in vitro conditions which affected the relative yields of ketone bodies and polycarboxylic acids respectively were the same whether octanoic acid or pyruvate served as substrates. On the basis of these findings it would appear that a common oxidation product is formed from both compounds. A view which has been widely accepted is that an acetyl-like two carbon unit forms the intermediate step at which the pathways of carbohydrate and fat metabolism converge.

However, certain difficulties are encountered when an attempt is made to correlate the ketone body formation from pyruvate *in vitro* with the metabolic fate of fat and carbohydrate in the intact organism. It is well known that in the starving or diabetic animal fatty acids enhance ketone body formation, whereas pyruvate or lactate reduce ketosis. Evidently, under these conditions, the two substances do not enter a common path. It is possible that acetoacetate formation from pyruvate is the favored reaction under special conditions in vitro, but it may still be quantitatively insignificant for the pyruvate metabolism of the

intact animal. The hypothesis that a C_2 fragment arises in the oxidation of both carbohydrate and fat would seem to be untenable unless it is assumed that the C_2 fragments are not identical.

The contrasting behavior of labeled fatty acids and pyruvate in the acetylation of foreign amines in intact animals leads to similar conclusions (30). While acetate is an effective source of acetyl for both aromatic amines (sulfanilamide) and α-amino acids (phenylaminobutyric acid), alanine (pyruvic acid) can yield acetyl to foreign α-amino acids only. Moreover, labeled alanine, unlike acetate or butyrate, does not serve as a precursor for cholesterol, either *in vivo* (30) or *in vitro* (97). If pyruvate were converted to the same C_2 fragment which is active in the acetylation of foreign amines and in cholesterol formation, labeled alanine or pyruvate should show the same effect as acetate. Recent experiments by Sonne et al. (98) on uric acid formation contain similar evidence. The feeding of $CH_3C^{13}OOH$ resulted in the incorporation of heavy carbon at carbon atoms 2 and 8 of the purine nucleus. No significant concentrations of C^{13} appeared at these positions when $C^{13}H_3C^{13}HOHCOOH$ was fed, indicating that lactate is not broken down to the two carbon compound employed in uric acid synthesis.

It is conceivable that the differences observed are quantitative rather than qualitative, but it is not likely that the bulk of pyruvate is oxidized by way of the same C_2 fragment which arises in the catabolism of fatty acids. Bloch and Rittenberg have concluded that the amounts of acetate produced in the intact rat can be accounted for fully by the oxidative metabolism of fatty acids and ketogenic amino acids (30). These data also would seem to exclude any considerable breakdown of pyruvic acid by way of acetyl as an intermediate.

Acetylmethylcarbinol (acetoin) has been obtained as a product of the anaerobic metabolism of pyruvate in micro-organisms (99) and in animal tissues (heart muscle) (100). The process is believed to involve oxidative decarboxylation to acetaldehyde, condensation of the latter with a second molecule of pyruvic acid, and subsequent decarboxylation of the condensation product. A scheme involving the following steps: pyruvate → acetoin → diacetyl → acetyl phosphate → acetate—has been proposed by Doisy and Westerfeld (24) on the basis of the effect shown by acetoin in the acetylation of p-aminobenzoic acid. The relatively high toxicity of acetoin for animals (101) and the fact that it is in part excreted after reduction to butyleneglycol (102) seems to preclude the possibility that the above scheme represents a major pathway of pyruvate metabolism.

The formation of acetate from pyruvate by an indirect route involving the splitting of intermediates of the tricarboxylic acid cycle has become a possibility in view of the following observations. The fermentation of citrate by yeast (103) and by various bacteria (104, 105, 106) has been found to yield 1 to 2 moles of acetate and smaller amounts of formate and succinate. These results have been interpreted to suggest the following series of reactions: citrate → acetate + oxaloacetate → pyruvate + CO_2 → acetate + formate. The first of these steps, i.e., the reversal of the condensation of acetate and oxaloacetate is believed by Lipton and Barron (107) to be responsible for the positive effect shown by citrate in the acetylation of choline in brain extracts. The reversibility of the initial

condensation reaction in the tricarboxylic acid cycle has not been tested in other systems of animal origin.

Slade and Werkmann (108) have studied the conversion of succinate to acetate by *Aerobacter indologenes* with the aid of C^{13}. Although these authors conclude that their data indicate a splitting of succinate into two molecules of acetic acid, it is equally possible that the acetate arose not from succinate directly but by degradation of either α-ketoglutarate or of a tricarboxylic acid formed from succinate. Irrespective of the pathway involved, these results suggest a possible route for the conversion of carbohydrate intermediates to acetate and fatty acids which circumvents the decarboxylation of pyruvate itself.

UTILIZATION OF ACETIC ACID FOR SYNTHETIC REACTIONS. The administration of labeled acetic acid to animals has been found to result in the incorporation of isotope into a variety of tissue constituents: glycogen, cholesterol, fatty acids, the dicarboxylic amino acids, protoporphyrin and uric acid. It thus appears that in the biological formation of the cell constituents acetic acid is of general importance as a source of carbon atoms. It has become increasingly evident in recent years that body constituents of high molecular weight are synthesized by condensation of numerous small sized units rather than by the utilization and rearrangement of preformed large molecules.

In a few cases only the animal organism depends exclusively on an exogenous supply for the maintenance of cellular composition (vitamins, essential amino acids and essential fatty acids). The majority of the tissue constituents can be derived not only from unchanged dietary material but can also arise by synthesis from small molecular breakdown products which are formed in intermediary metabolism. The ability of the animal organism to keep its tissue composition constant under a variety of dietary conditions is an expression of the fact that the required elementary building stones are available from a number of sources.

Older suggestions that cholesterol might be formed either by modification of the preformed steroid structure which is offered to the animal in the form of squalene (109) or plant steroids (110), or by folding of long chain fatty acids (111) have given way to the view that a total synthesis from numerous small units is involved (112). In the synthesis of long chain fatty acids from carbohydrate the sugar molecule is presumably not utilized as such but only after its degradation to C_2 or C_3 fragments. In the formation of the tetra pyrrole structure of porphyrin, two C_2 compounds, acetic acid (30) and glycine (113, 114), serve as a source of carbon and nitrogen and not the preformed five-membered ring structures of proline or pyrrolidonecarboxylic acid (from glutamic acid). The investigations on the biological synthesis of these high-molecular tissue constituents have so far been carried out mainly with intact animals. It is evident that for the synthesis of the more complex molecules a high state of cellular organization will be required. For this reason virtually no attempts have been made to investigate the enzymatic processes concerned in the synthesis of the substances mentioned. It has not been possible to identify any of the intermediate steps in the biosynthesis of fatty acids, cholesterol or porphyrin, nor do indications exist that such intermediates occur normally in measurable quantities.

The utilization of acetic acid for biological syntheses has been demonstrated by the feeding of labeled acetic acid to animals and isotope analysis of the body constituents isolated from the tissues. Insight into the reaction mechanisms has in some cases been gained by determining the location and distribution of isotope at various positions of the synthetic product, but the accessible information is often limited by the lack of suitable methods of degradation. Wood et al. (115) have employed chemical and microbiological degradation procedures for glucose which permit separate isotope analysis of carbon atoms 3 and 6 and of fractions which represent carbon atoms 1 and 6, 2 and 5, and 3 and 4 respectively. Methods suitable for determining the deuterium concentrations at separate positions of deuterioglucose have been described by Stetten and Stetten (116). Although procedures are available for successive degradation of the higher fatty acids they are not applicable to small quantities of biological material. However, analysis of the carboxyl group and of the two moieties obtainable by degradation of oleic acid have yielded valuable information on the mechanism of fatty acid synthesis (117). The distribution of isotope in deuteriocholesterol has been determined by degradation of the steroid molecule into nucleus and side chain (118). No experiments of this type have so far been carried out with protoporphyrin formed biologically in the presence of labeled acetate.

Although it may be feasible to study details of reaction mechanisms by employing test substances which contain more than one isotopic marker, there are certain limitations to the experimentation with intact animals. If, in a synthetic process, acetic acid did not take part as such but in the form of a functional derivative (acetylphosphate, acetylamine) and if the substituent were eliminated in the course of the condensation, isotope analysis of the reaction product may not reveal whether acetic acid reacted as such or in a combined form. On the other hand, experiments with intact animals are indispensable in order to establish the occurrence and the quantitative significance of a process which has been observed in an isolated system.

Glycogen. According to classical concepts, a glycogenic compound is a substance which either increases glycogen deposition in the liver of a fasted animal or enhances glycosuria in the diabetic animal. Acetic acid does not satisfy these criteria, since its feeding neither increases glycogen deposition nor glucose excretion (119). On the other hand, isotope experiments have shown that acetic acid can provide carbon atoms for all positions of the glucose molecule (120, 121). No inconsistency need be seen in these findings, if (a) the meaning of the term precursor is more clearly defined and if (b) it is recognized that the terms glycogenic and ketogenic apply only to special experimental conditions. The revision of classical concepts necessitated by the results obtained with tracer substances has been discussed by Buchanan and Hastings (122) and by Wood (123). If one disregards reactions in which a substance effects an increased formation of a product by stimulation rather than by direct conversion, then two types of precursors have to be considered: substances which afford a net increase of the quantity of product, i.e., reactions in which the quantity of precursor is the limiting factor, and secondly substances which are incorporated either totally or

in part into reaction products but which are without effect on the rate of its formation. Compounds which in classical terminology are classified as either glycogenic or ketogenic belong to the first group while acetic acid with respect to glycogen formation belongs to the second category. Although, as Lorber, Lifson and Wood (121) have shown, acetic acid provides carbon atoms for all positions of the glucose molecule, it probably cannot by itself form hexose (or pyruvate) but only by entering the tricarboxylic acid cycle. Through a series of transformations the tricarboxylic acid yields pyruvate, which is the obligatory intermediate in glucose formation. Acetate utilization for glycogen synthesis is therefore not regulated by the availability of acetic acid but depends on tricarboxylic acid formation and regeneration of pyruvate from the latter. On the other hand the conversion of pyruvate to glycogen does not require the intervention of the citric acid cycle and as a result pyruvate and compounds which are directly convertible to pyruvate will be precursors of glycogen in the sense that they can increase the net amount of glycogen formed. It is evident that the terms glycogenic and ketogenic apply only to conditions in which a change of carbohydrate concentration is observable, i.e., in the starving or diabetic animal. From its behavior in isolated liver, pyruvic acid could be classified as a ketogenic substance, since under such conditions it can be converted in part (124, 33, 90, 96) or quantitatively (125) to acetoacetatic acid. Acetic acid is ketogenic since it increases ketone body excretion in the starving or diabetic animal (5), but as isotope studies have shown, it is also a source of carbon atoms for glucose. The appearance of acetate carbon in all positions of the glucose molecule has been ascribed by Wood (115) to a series of steps by which a tricarboxylic acid formed from acetate and oxalacetate is degraded to pyruvate by way of α-ketoglutarate and the C_4 dicarboxylic acids. If $C^{13}H_3C^{13}OOH$ is employed the resultant pyruvate will be labeled at all three positions but it cannot contain more than half of the isotope concentration present in the acetate which was utilized. On tracing the labeled carbon through tricarboxylic acid, α-ketoglutarate and the dicarboxylic acids to succinate, it becomes clear that only two of the four carbons in the dicarboxylic acids originate from acetate but the isotopic carbon becomes evenly distributed over the entire carbon chain when the asymmetry of the molecule is lost at the succinate stage. Pyruvate will therefore contain C^{13} at all positions. It can be concluded that although acetate carbon appears in all positions of glucose not more than half of the carbon atoms of a glucose molecule can have originated from acetate, the remainder being supplied by oxaloacetate. Acetate per se is therefore incapable of forming glucose.

Carboxylation of acetic acid to pyruvic acid cannot be an important pathway for the utilization of acetate in glycogen formation because in this case the isotope distribution in glycogen would be expected to differ from that actually observed (121).

Fatty acids. McLean and Hoffert (126) have studied lipid formation in yeast from acetate and have concluded from balance data that both steroids and neutral fat could be synthesized directly from acetate without intermediate formation of carbohydrate. In the presence of sulfite steroid synthesis was

inhibited, but fat continued to be synthesized. This suggested that acetaldehyde might be an intermediate in the synthesis of yeast steroids while acetate could condense directly to fatty acids. In their experiments on the metabolism of deuterioacetate in yeast, Sonderhoff and Thomas (127) found the following concentrations of deuterium in the various fractions: carbohydrate 1.6 per cent; fatty acids 14.7 per cent; unsaponifiable 30 per cent, indicating that carbohydrate was not an intermediate in the conversion of acetate to lipids.

Wood et al. (128) have demonstrated the bacterial utilization of acetic acid for the synthesis of lower fatty acids. In the presence of $CH_3C^{13}OOH$, the butyl alcohol formed in the fermentation of *Cl. butylicum* and of *Cl. acetobutylicum* was found to contain C^{13} at carbon atoms 1 and 3. Since these organisms can reduce butyric acid to butanol, the following series of reactions is suggested: 2 acetate $\rightarrow$ butyrate $\rightarrow$ butanol.

The fermentation of alcohol and acetate in *Cl. kluyveri* has been studied by Barker, Kamen and Bornstein (129). The butyric acid formed in the presence of $CH_3C^{14}OOH$ contained approximately equal quantities of heavy carbon in the carboxyl and β positions; in caproic acid equal amounts of C^{14} were presumably present at alternate carbon atoms since the carboxyl group accounted for about one-third of the total isotope content of the hexanoic acid. Important information with respect to the mechanism of fatty acid synthesis in these organisms was obtained by the same authors. Isotopic caproate formed in the presence of $CH_3CH_2CH_2C^{14}OOH$ and normal ethanol contained isotope but this was not present in the carboxyl group. The active C_2 unit derived from ethanol must therefore have condensed with the carboxyl group of butyric acid. Moreover, a breakdown of butyrate to C_2 compounds prior to formation of the C_6 acid cannot have occurred since in this case the carboxyl carbon of caproic acid should have contained the carbon isotope.

Rittenberg and Bloch (117) have fed mice and rats acetic acid which contained C^{13} at the carboxyl position and deuterium in the methyl group. The fatty acids isolated from the tissue lipids contained both isotopes in excess of the concentrations which could have resulted from the oxidation of acetate to $C^{13}O_2$ and D_2O and incorporation of deuterium from the body fluids or C^{13} by a reaction involving CO_2 assimilation. The body fluids of the mice contained 0.09 per cent D and the respiratory CO_2 contained 0.066 per cent C^{13} while the saturated fatty acids isolated from the liver lipids contained 0.42 per cent D and 0.16 per cent C^{13}. As the fatty acids contained both C^{13} and deuterium it is clear that the acetic acid molecule as such had been utilized in the synthetic process. From acetic acid containing isotopic carbon in the carboxyl group, a fatty acid containing C^{13} only at one out of two carbon atoms is to be expected. The incorporation of isotope into the fatty acids could result from the following processes: 1, acetic acid elongates the fatty acid chain by adding at the carboxyl end. In this case the presence of isotopic carbon should be confined to the carboxyl carbon of the fatty acid and decarboxylation should leave a non-isotopic residue; 2, If acetic acid condensed with the ω carbon of the fatty acid decarboxylation should yield CO_2 which contains no excess of isotope; 3, acetate carbon is uniformly

distributed over the fatty acid chain. C^{13} should then be present at the odd-numbered carbon atoms. The CO_2 obtained on decarboxylation of the fatty acid actually contained about twice as high an isotope concentration as the entire molecule, indicating that C^{13} was present at alternate positions, i.e., at the odd-numbered carbon atoms, one of which is the carbon of the carboxyl group. Additional support for the view that the acetic acid carbon was distributed in random fashion over the entire fatty acid chain is afforded by analysis of the degradation products of the "oleic acid" fraction of tissue fat. This consists mainly of oleic acid and some palmitoleic acid and yields on oxidation a monocarboxylic acid, pelargonic acid, derived from carbon atoms 10 to 18 and a dicarboxylic acid (azelaic acid) representing carbon atoms 1 to 9. If a C_{18} fatty acid were formed from nine acetate units then of the nine isotopic carbons, five should be present in the azelaic acid and four in the pelargonic acid fraction, while the deuterium content of the two moieties should be nearly equal. The following isotope concentrations were found: azelaic acid, 0.090 per cent C^{13}, 0.14 per cent D; pelargonic acid, 0.071 per cent C^{13}, 0.15 per cent D. These data suggest a process of fatty acid synthesis involving the multiple condensation of C_2 units. Any scheme proposed as a mechanism of fatty acid synthesis must account for the fact that the fatty acids of animal tissues have an even number of carbon atoms and comprise, if milk fat is included, all members of the series from C_4 to C_{24}. The occurrence of C_2 condensation in the synthesis of fatty acids by animal tissues was first demonstrated by the finding of Stetten and Schoenheimer (130) that deuteriostearic acid is formed from deuteriopalmitic acid by chain elongation.

The reactive two carbon compound undergoing the condensation can be readily formed from acetic acid in the intact animal and in bacterial systems, but its chemical identity has not been determined. Fatty acid synthesis in animal cells has so far not been demonstrated in isolated systems. When rat liver slices were incubated with labeled acetic acid only very small amounts of isotope were incorporated into the fatty acids; significant concentrations of deuterium were found in the liver fatty acids when the buffer medium contained D_2O (131). From this result it cannot be decided whether the uptake of isotope resulted from synthesis of the carbon chain or whether it was due to hydrogenation of unsaturated fatty acids.

The production of C_2 units in the course of fatty acid oxidation and their utilization in fatty acid formation points to a mechanism of synthesis which consists of the reversal of the catabolic steps. This would involve the condensation of two carbon units with acetate and higher fatty acids and the subsequent reduction of β keto compounds to saturated acids. The well established conversion of acetate to acetoacetate may represent the initial step in this process. However, the biological reduction of β-keto acids to the corresponding saturated fatty acids has never been shown to occur.

Experiments with acetic acid containing deuterium as well as C^{13} have thrown some light on the nature of the intermediates involved in fatty acid synthesis (117). When a preparation of acetate was fed in which deuterium and carbon

were present in a ratio of 8:1 the fatty acids isolated contained the two isotopes in a ratio of 2.5:1. Irrespective of the nature of the condensation, there should be introduced for every two deuterium atoms from the methyl group of acetic a minimum of two atoms of ordinary hydrogen from the body fluids. The ratio of $D:C^{13}$ should then become 4:1. The greater loss of D than corresponds to this ratio may have resulted from keto-enol tautomerism of the intermediate β-keto acid. An additional atom of normal hydrogen from the body fluids would also be introduced if the keto acid were converted to the saturated acid by way of the hydroxy- and unsaturated acids.

Although the isotope data suggest that acetic acid can supply the carbon atoms for all positions of the fatty acid chain, it is unlikely that acetate is a precursor in the sense that its administration produces a net increase of synthesized fat. Experimental conditions for studying fat synthesis from specific precursors, similar to those by which the glycogenic or ketogenic properties of a compound can be tested, are not available. Conditions which result in the deposition of excessive quantities of fat in the liver such as choline deficiency do not measure changes in the amount of fat synthesized but reflect deficiencies in fat transport. Stetten and Boxer found that in rats treated with alloxan, fat deposition and incorporation of deuterium from heavy body water are greatly reduced (132). This may be the result of diminished fat synthesis or of a failure to deposit the newly formed fat. The impairment of fatty acid synthesis in this condition is probably not due to a lack of precursor but may reflect, as Stetten and Boxer suggest, a general inability to utilize carbohydrate. Since in the experiment of Rittenberg and Bloch (117) the animals received a fat free diet, carbohydrate and protein must have provided the carbon for fatty acid synthesis. It was estimated that under these conditions about 25 per cent of the carbon atoms of the fatty acids were derived from acetate. While the conversion of carbohydrate to fat is well established from balance experiments, no information is available concerning the intermediate steps. It is generally assumed that synthesis of the fatty acid chains occurs with pyruvate or a two carbon compound derived from pyruvate. It is questionable whether this intermediate is identical with acetic acid because in this case not only one-fourth but all carbon atoms of the fatty acids should be available from the "acetyl" pool. It has been pointed out above that pyruvic acid or alanine are not convertible to the acetyl compound involved in the acetylation of sulfanilamide or in the formation of cholesterol. This has been taken to mean that a direct degradation of pyruvic acid to a C_2 compound does not occur or that the product is not identical with the acetyl formed by fatty acid oxidaton or from acetate itself.[3] In order to allow for the participation of both acetic acid and carbohydrate in fatty acid synthesis, Rittenberg and Bloch (117) have suggested that pyruvate may form acylpyruvic acids and that the decarboxylation to β-keto acids takes place after the condensation

[3] This conclusion was based on experiments with deuterio alanine. It does not take into account the possibility that pyruvate is converted to acetyl by a circuitous route in which deuterium is lost entirely. Recent experiments of Sonne et al. (98) on uric acid synthesis with test substances containing C^{13} similarly indicate that lactate is not converted to acetate.

$$RCH_2COOH + CH_3COCOOH \rightarrow RCH_2COCH_2COCOOH \rightarrow RCH_2COCH_2COOH + CO_2 \quad [9]$$

In this reaction the net effect is the addition of a two carbon unit, without acetic acid appearing as an intermediate. It is also conceivable that an intermediate of the tricarboxylic acid cycle which contains the elements of both acetic and pyruvic acids supplies the C_2 fragments for fatty acid synthesis. For instance, the C_4 dicarboxylic acids which are derived from the condensation product of acetate and oxaloacetate are composed of two C_2 moieties one of which originates from acetate and the other from pyruvate. By passing through a symmetrical stage, randomization would result, and in a utilization of the dicarboxylic acid for fatty acid synthesis an equal chance would exist that the C_2 fragment was contributed by either acetate or pyruvate.

It has been pointed out above that the acetic acid arising in intermediary metabolism appears to be derived primarily from the catabolism of fatty acids and ketogenic amino acids while carbohydrate does not contribute significantly to the acetate pool. It would follow, then, that the acetic acid employed for fatty acid synthesis has its origin in the fatty acids themselves; in other words, the process of acetate utilization merely reflects the continuous molecular regeneration of body fats. Since a large portion of the acetyl fragments arising in fatty acid oxidation is metabolized further and is therefore unavailable for fat resynthesis, carbon has to be provided from another source in order to keep the total quantity of fat constant. The source is evidently carbohydrate. Acetate is therefore not a precursor of fatty acids in the conventional sense and the statement that 25 per cent of the carbon atoms are supplied by acetate merely implies that one out of every four carbon atoms incorporated into a newly synthesized fatty acid molecule arose from the catabolism of the fatty acids themselves.

Cholesterol. In their experiments on the utilization of deuterioacetate by yeast Sonderhoff and Thomas found the deuterium concentration in the unsaponifiable fraction to be twice as high as in the fatty acid fraction and twenty times that of the yeast carbohydrate (127); they therefore suggested that in the synthesis of the yeast steroids acetate had been employed directly. A formation of cholesterol in animal tissues by total synthesis from small units was indicated by the findings of Rittenberg and Schoenheimer (112). In cholesterol newly formed by animals whose body fluids were enriched with heavy water roughly half of the hydrogen atoms were derived from the D_2O of the body fluids. The utilization of various small molecular substances for cholesterol synthesis has been investigated by Bloch and Rittenberg in feeding experiments with labeled compounds (133, 118, 78). The isotope concentration in cholesterol isolated from the tissue lipids was highest when labeled acetic acid was the test substance. Whenever another compound was effective in causing the formation of labeled cholesterol the result could be attributed to intermediary formation of acetate from the test substance (butyrate, ethanol, leucine, valerate, isovalerate). By studying simutaneously cholesterol synthesis and the acetylation of foreign amines it could be shown that the ability of a test substance to cause the formation of

labeled cholesterol was roughly parallel with its ability to furnish labeled acetyl groups. This correlation did not exist in the case of alanine. The significance of this finding has been discussed above. An evaluation of the quantitative rôle of acetic acid as a precursor for the steroid structure led to the conclusion that about half of the hydrogen atoms (and probably half of the carbon atoms) are furnished by acetate. Acetic acid appears to contribute to the formation of the entire steroid molecule. When cholesterol formed biologically from deuterio acetate was degraded, the iso-octyl fragment derived from the cholesterol side chain and the hydrocarbon representing the steroid skeleton contained deuterium in nearly equal concentrations. No information is available as to the intermediate steps of the synthetic process. The higher fatty acids or intermediates of fatty acid metabolism cannot lie on the path of the acetate-sterol conversion, since in feeding experiments with deuterio acetate, newly formed cholesterol contains several times the isotope concentration of the fatty acids; also, in liver slices deuterium is rapidly incorporated into cholesterol when labeled acetate is present, but not into fatty acids (131). The utilization of both carbon atoms of acetic acid is evident from the appearance of both deuterium and C^{13} in cholesterol synthesized from $CD_3C^{13}OOH$ (117, 131).

Since the acetate-cholesterol conversion entails essentially the transformation of—$CD_2 \cdot CO$—into—$CD_2 \cdot CH_2$—two normal hydrogen atoms should be incorporated for every two carbon-bound deuterium atoms and in the utilization of $CD_3C^{13}OOH$ the ratio of D to C^{13} should decline to half its original value. However, the relative amounts of D and C^{13} introduced into cholesterol were roughly the same as in the administered acetate and it would appear therefore that the reaction product contained, perhaps as the result of decarboxylations, more of the methyl carbons than of the carboxyl carbons of acetic acid.[4]

Some indications exist that a reduction may be the initial step in the acetate-cholesterol conversion. McLean and Hoffert (126) observed that sterol synthesis from acetate in yeast was inhibited by sulfite, a reagent which blocks aldehyde groups. In the formation of labeled cholesterol by rat liver slices, labeled ethanol and acetaldehyde were somewhat more effective than acetic acid (97) suggesting that the intermediate is more readily formed from ethanol and acetaldehyde. It is unlikely that the somewhat higher efficiency of acetaldehyde and ethanol under these conditons is due to the fact that they diffuse more readily into the liver cell since the deuterium concentration in cholesterol is independent of the acetate concentration in the buffer medium over a wide range (131).

In the intact animal and in liver slices deuterio alanine fails to give labeled cholesterol (118, 97). Alanine, and presumably pyruvic acid, are therefore not convertible to the C_2 compound which provides carbon for cholesterol synthesis and which is readily formed from acetic acid itself and from the higher

[4] The results obtained with intact animals differ from those obtained in liver slices. The ratio of D to C^{13} in cholesterol formed from acetate in vitro changes from 8 to 2.5. This might be explained by the loss of carbon bound deuterium associated with the interconversion of acetate and acetoacetate which proceeds rapidly in liver slices but apparently not in the intact animal.

fatty acids. This finding supports the previously expressed view that if a C_2 fragment arises in carbohydrate oxidation it is unlikely to be identical with that derived from fatty acids.

The origin of the remaining carbon and hydrogen atoms in cholesterol, which are not accounted for by acetic acid, is unknown. In view of the fact that cholesterol contains a branched side chain and angular methyl groups and that these groupings are otherwise present only in dietary constituents which animals cannot synthesize (valine, leucine, carotene), the possibility has been tested that one of the branched chain amino acids participates in steroid synthesis. The feeding of leucine which contained deuterium at all positions of the carbon chain resulted in the formation of labeled cholesterol while similary labeled valine was ineffective (78). However, leucine is a ketogenic amino acid and in the course of its catabolism yields acetyl groups. Hence, no conclusions may be drawn as to whether leucine specifically supplies the branched side chain and the angular methyl groups of cholesterol or whether the observed effect is ascribable merely to the intermediary formation of acetic acid.

Cholesterol can serve as the parent substance for other compounds which possess the cyclopentano-phenanthrene structure. The in vivo transformation of cholesterol into cholic acid (134) and into pregnanediol (135) has been demonstrated with the aid of deuteriocholesterol and it has been found that a minimum of two-thirds of these steroids can arise by degradation of cholesterol. It is therefore not too likely that animal steroids other than cholesterol can also be formed by total synthesis from small units by a pathway in which cholesterol is not an intermediate.

Protoporphyrin. Hemin isolated from the red cells of animals which receive deuterio acetic acid contains deuterium in significant concentrations (30). In short term feeding experiments the isotope content of hemin will attain only a relatively low level, since the protoporphyrin moiety of hemoglobin is synthesized at a much slower rate than most body constituents (114). When deuterioacetate feeding is extended to a period of several weeks the deuterium concentration in hemin reaches a value corresponding to 50 per cent of the isotope concentration in the acetic acid available in the metabolic pool (136). Therefore at least half of the hydrogen atoms in the porphyrin molecule are supplied by acetic acid. The constituent pyrrol nuclei in the porphyrin molecule contain no hydrogen linked to carbon; carbon bound hydrogen is present in the methin bridges which link the pyrrol rings and in the pyrrol side chains. Hence, experiments with deuterio acetic acid do not reveal whether acetic acid is also a source of carbon atoms for the pyrrol rings. A mechanism of synthesis which leads directly to a pyrrol structure containing the appropriate side chains would seem to be a more likely event than a process in which the substituent side chains are attached to a preformed pyrrol nucleus. No deuterium is introduced into hemin after the feeding of deuterio propionic acid while β-γ-dideuterio butyric acid is about half as effective as deuterio acetic acid (136). The effect shown by butyrate is most likely due to its splitting into acetyl fragments.

The origin of pyrrol nitrogen has been elucidated by the experiments of

Shemin and Rittenberg (114, 137). Hemin isolated from the blood of a human subject who had received glycine labeled by N^{15} contained so high an isotope concentration as to indicate a specific utilization of glycine nitrogen. The specific role of glycine in pyrrol synthesis received further support from the finding that glycine was the most effective source of pyrrol nitrogen among several amino acids tested (114). In particular, proline and glutamic acid, which have been suggested as possible biological precursors of pyrrols because they contain or can readily form heterocycles similar to pyrrols, were much less effective as sources of nitrogen for hemin than glycine. The utilization of the two carbon compounds, acetic acid and glycine, points to a mechanism of porphyrin synthesis in which the ring structure and the substituents are formed simultaneously, possibly by processes analogous to those which yield pyrrols in organic chemistry. Fischer and Fink (138) have obtained evidence that ring closure to a pyrrol occurs when glycine and formylacetone (the aldehyde of acetoacetic acid) react in aqueous solution at 37°. The classical pyrrol synthesis of Knorr (139) involved the condensation of α-amino acetoacetic ester with acetylacetone. Acetic acid can be converted to aceto-acetate in vivo and it is not inconceivable that by an analogous process α amino-acetoacetic acid is formed biologically from acetic acid and glycine. A biological pyrrol synthesis analogous to the Knorr reaction would be attractive because it would afford a product in which the arrangement and the chemical nature of the substituents resembles that in the naturally occurring protoporphyrin.

Uric acid. Recently the utilization of C_2 compounds for the synthesis of still another group of tissue constituents has been reported. Sonne, Buchanan and Delluva (98) have studied the incorporation of C^{13} into uric acid, isolated from pigeon excreta, after the feeding of $CH_3C^{13}OOH$, $CH_3^{13}C^{13}HOHCOOH$, $CH_3CHOHC^{13}OOH$ and $NH_2CH_2C^{13}OOH$ respectively. The carboxyl carbon of acetic acid was located at the two ureido carbons (2 and 8) of the purine structure, while the carboxyl group of glycine supplied carbon for position 4, and CO_2 carbon for position 6. On the basis of the data available so far no plausible mechanism for purine synthesis from CO_2, acetate and glycine can be suggested. It is of interest in this connection that Barker and Beck (140) found ammonia, carbon dioxide, acetic acid and small amounts of glycine to be formed as products of uric acid fermentation by *Clostridium acidi urici*. The findings of Sonne et al. contain important evidence with respect to some other metabolic processes. The appearance of acetate and glycine carbon at different positions of the uric acid eliminates the possibility that in the pigeon glycine and acetic acid are interconvertible. On the basis of nutritional studies, Almquist et al. had contended that the chick is unable to synthesize adequate amounts of glycine and that in deficient diets acetate may serve as substitute for glycine (141). The interconvertibility of the two compounds which is implied by these findings is contradicted by the tracer experiments of Sonne et al. unless metabolic differences exist between the chick and the pigeon.

The isotope data obtained by Sonne et al. furthermore show that the α carbon of lactate is not incorporated into the same positions of uric acid as the carboxyl

carbon of acetate. A breakdown of lactate to acetate did, therefore, not occur to any considerable extent. These results are incompatible with the assumption that the hypothetical two carbon compound arising in pyruvate oxidation is closely related to acetic acid.

A relationship between acetate and lipid metabolism in bacteria has been observed by Guirard, Snell and Williams (142). Acetate stimulates the early growth of lactic acid bacteria, an effect which is also shown by fatty acids and various steroids. These results suggest an important rôle of acetate in the synthesis of lipids by bacterial systems.

As the result of investigations with isotopically labeled test substances there have been developed a number of novel biochemical theories among which Schoenheimer's concept of the dynamic state of the body constituents has been the most outstanding (143). According to this concept, there exists a pool of metabolites which is continuously replenished by the breakdown products of both dietary and tissue constituents and which supplies the metabolites for energy production and for the rebuilding of the structural components of the cell. The concept of the metabolic pool was advanced in order to interpret the data obtained from feeding experiments with isotopic substances and in order to provide a reasonable basis for the rapid interchange of dietary and tissue elements which was indicated by these results. It should be pointed out that the metabolic pool may not possess any physical reality in the sense that there exists in the body at a given moment a large reservoir of metabolites in which molecules derived from dietary and various tissue constituents are distributed in random fashion and are therefore undistinguishable. It is merely permissible to say that if for example 100 molecules of dietary origin enter the tissues during a given period of time and 100 molecules arise from body constituents during the same interval, then the chances that in a subsequent reaction either the exogenous or endogenous molecule is employed will be equal. The metabolic pool should therefore be regarded merely as an expression of this probability.

One of the problems of metabolism which has held the general interest of the biochemist has been the mutual interconversion of fat, carbohydrate and protein. These questions have only partially been answered by balance techniques in which the criterion of a biochemical conversion is the net change in amount of the reaction product which is induced by the precursor. In the light of the concept of the metabolic pool, biochemical interconversions assume a different meaning. Fat, protein and carbohydrate are interconvertible in the sense that they give rise to breakdown products which lose the identity of their origin and can be utilized interchangeably for the resynthesis of either one of the three major tissue constituents.

The fact that acetic acid is employed as a building stone in the synthesis of a variety of compounds of different chemical structure and function, viz. lipids, carbohydrate, porphyrin, uric acid and some of the amino acids, suggests its direct or indirect participation in the biological formation of all compounds which can be synthesized by the animal cell. If this is true then acetic acid cannot be regarded as a specific precursor of a particular tissue constituent, but its utiliza-

tion would reflect merely the fact that the body employs two carbon units as a principal source for synthetic reactions.

The biologically active form of acetic acid. Since in isolated tissues acetic acid shows less reactivity than in the intact animal the possibility has been widely discussed that acetic acid is metabolically converted into a more reactive form. In organic chemistry such activations are achieved by esterification or anhydride formation (acetylchloride, acetic anhydride, ketene, ethylacetate).[5]

Experimental evidence for the biological formation and the participation in a synthetic reaction of an acetic acid derivative rather than of acetic acid itself has been provided in two instances. In bacterial systems the phosphoroclastic splitting of pyruvate to acetylphosphate and formate can be reversed (145). No other function for acetylphosphate has been found so far. A process of intermolecular acetyl transfer in which acetyl groups are shifted from an N-acetyl amino acid to a free amino acid has been indicated by acetylation studies in the intact animal (35).

There are two other well known biochemical processes which are classified as transfer reactions, transfer of methyl groups involving choline, methionine and creatine (146) and the amidine transfer from arginine to glycine in the formation of guanido acetic acid (147, 148). Such reactions cannot be described in terms of ordinary chemical equations involving stable compounds as intermediates. It is conceivable that group or radical transfer may be a more general phenomenon in biological systems and that the answer to the acetyl problem may be sought in this direction.

THE OXIDATIVE METABOLISM OF ACETIC ACID. The ability of animal tissues to utilize acetic acid efficiently is illustrated by the fact that when fed to animals acetic acid does not appear in the urine in measurable amounts. In the experiments of Buchanan, Hastings and Nesbett with acetate containing radioactive carbon in the carboxyl group, over fifty per cent of the acetate absorbed by the rats was totally oxidized within two hours, as determined by the appearance of isotopic carbon in the expired carbon dioxide (120). This rate was comparable to that at which similar quantities of lactic acid were metabolized (149).

Acetate disappears aerobically from a variety of isolated tissues, the rate being most rapid in the cortex of guinea pig-kidney (150). Little evidence exists that the acetate molecule as such can undergo oxidative reactions.[6] Products which could conceivably arise by oxidation of the methyl group of acetic acid such

[5] For a consideration of the "active" form of acetic acid the following organic chemical reactions are of interest. The studies by Bergmann et al. (36) on intermolecular transfer of acetyl groups or radicals between amino compounds have been mentioned above. Kharasch and Gladstone (144) have shown that by conversion into diacetyl peroxide, the methyl groups of acetic acid become sufficiently activated to undergo α-α condensation to succinic acid. The reaction is believed to be due to the dimerization of an intermediate CH_2COOH radical.

[6] The claim of Toenniessen and Brinkmann (151) that formate is a product of acetate metabolism in perfused muscle is based on insufficient experimental evidence, but conforms with recent observations of Buchanan and Sonne (167) on the rôle of acetate and formate respectively as precursors of uric acid.

as glycolic, glyoxylic, oxalic acids or glycine are metabolically inert under conditions which permit of rapid oxidation of acetate (152). Many years ago Thunberg suggested that the oxidation of acetate was initiated by the dehydrogenative coupling of the methyl groups of two molecules of acetate to succinic acid (55).

$$HOOC\cdot CH_2\boxed{H + H}CH_2COOH \longrightarrow HOOC\cdot CH_2CH_2COOH \quad [10]$$

The same reaction was proposed later by Wieland and Sonderhoff to account for the formation of succinate by yeast in which acetate was the sole nutrient (153). Slade and Werkmann (108) have studied this process with C^{13} containing acetate in *Aerobacter indologenes* and have concluded that the isotope data demonstrate methyl condensation of two moles of acetate. Lipmann has suggested that this condensation may involve acetylphosphate (154). The inhibition by malonate of acetate and acetoacetate disappearance in animal tissues (155, 156) and of acetate in yeast (157) has been cited in support of reaction [10] but the fact that acetate oxidation is sensitive to malonate may merely be taken to suggest that succinic acid lies on the path of acetate metabolism; it does not indicate the mechanism involved. There is increasing evidence that the step which precedes acetate oxidation consists of a condensation with a C_4 dicarboxylic acid. From the resultant C_6 tricarboxylic acid two carbon atoms are removed by oxidation to regenerate the dicarboxylic acid:

$$CH_3COOH + C_4 \rightarrow C_6 \rightarrow 2\,CO_2 + C_4 \quad [11]$$

The experimental support for this scheme coincides in many instances with the evidence which led originally to the formulation of the "citric acid" cycle for the metabolism of pyruvic acid. In fact, in some respects the rôle of the tricarboxylic acids is better understood for the oxidative phase of fatty acid and acetate metabolism than for the oxidation of pyruvate. Acetate and acetoacetate have been shown to form the intermediate polycarboxylic acids of the cycle, but it has remained undetermined whether pyruvate itself enters into combination with the C_4 dicarboxylic acids or whether the condensation is preceded by a degradation of pyruvate to a two carbon unit. The significance of the citric acid cycle in the metabolism of fat and carbohydrate respectively is still a matter of controversy. According to Breusch, carbohydrate is not oxidized to an appreciable extent by way of the citric acid cycle (158), while Weil-Malherbe (159) and Krebs (160) reject the view that the citric acid cycle is involved in the oxidation of acetoacetate.

Acetoacetate, acetate and pyruvate have been shown to be convertible, in the presence of dicarboxylic acids, to citrate and intermediates of the tricarboxylic acid cycle, but whether a single mechanism is invoved in all cases is undecided. However, the citric acid cycle constitutes the most successful attempt to date to describe the oxidative phases of fat, carbohydrate and protein metabolism by a unified scheme which visualizes a junction of metabolic pathways and leads to a common mechanism for the ultimate oxidation of the three major dietary and tissue constituents.

The citric acid cycle in relation to the metabolism of carbohydrate has been discussed in reviews by Werkmann and Wood (161), Krebs (162) and Evans (163). The citric acid cycle as a vehicle for fat oxidation has been considered in detail by Wood (123). The present discussion will be concerned primarily with experimental data which deal with the oxidation of acetic acid.

Since there is little evidence to support the assumption that the carbon chain of acetate as such is susceptible to oxidation the question arises as to the steps which precede the conversion of acetate to carbon dioxide and water. Breusch (158) has suggested that the intermediate β keto acids formed in fatty acid oxidation combine directly with oxalacetate to give a product which contains the two terminal carbon atoms of the fatty acid chain, leaving a fatty acid shortened by two carbon atoms. According to this view, two carbon compounds would not arise as distinct intermediates in fat oxidation. Since acetic acid is a compound of great chemical stability and, compared to other metabolites, relatively unreactive in some biological systems, the view has frequently been expressed (123) (164) that acetic acid itself may never arise but may be the stabilization product of a more reactive C_2 unit. The identity of this intermediate has not been established in animal tissues. Judging from the variety of reactions which it undergoes in the intact animal and in some isolated systems, acetic acid must be readily convertible to the hypothetical reactive C_2 fragment.

$C_2 + C_1$ addition. The reversal of the phosphoroclastic splitting of pyruvic acid to formate and acetylphosphate according to the equation:

$$CH_3COCOOH + H_3PO_4 \rightleftarrows CH_3COOPO_3H_2 + HCOOH(H_2 + CO_2) \quad [12]$$

is well established in bacterial systems (145). Lipmann has demonstrated convincingly that acetyl phosphate is the product of this bacterial dissimilation (165). The preservation of a large fraction of energy in the carboxylphosphate facilitates the reverse reaction, i.e., the carboxylation of the C_2 compound, a reaction which is energetically improbable with acetate itself. It has not been possible to demonstrate acetylphosphate as a product of pyruvate metabolism in animal tissues nor can the evidence for the formation of acetylphosphate from acetate in animal tissues be considered sufficient. Lipmann and Tuttle (166) have incubated extracts from pigeon liver with acetate and adenosine triphosphate and observed that on addition of hydroxylamine and ferric chloride a chromogen was formed which had the properties of hydroxamic acids obtainable from acetylphosphate or aldehydes, but more direct proof is desirable to establish the identity of the acetyl compound formed under these conditions. The reversal of reaction [12] is a formylation rather than a carboxylation and apparently CO_2 can be utilized only in organisms which hydrogenate carbon dioxide to formate. In considering the likelihood that reaction [12] occurs in animal tissues, it should be noted that so far no function has been assigned to formic acid in animal metabolism.[7] Experiments of Lorber et al. (121) in which the isotope distribu-

[7] This statement may have to be revised in view of the recent finding by Buchanan and Sonne (167) that formate is utilized in uric acid synthesis by pigeons. The authors suggest that formic acid may arise by oxidation of acetate.

tion in glucose was studied after feeding of labelled acetic acid contain no evidence for $C_2 + C_1$ addition. Carboxylation of acetic acid which contained C^{13} in the carboxyl group would yield pyruvate with the labeled carbon at the α carbon atom. If such pyruvate were further carboxylated to oxaloacetate and reformed after passing through the stage of a symmetrical dicarboxylic acid, the isotopic carbon would become equalized between the α and β positions of pyruvate. Glucose formed from the singly labeled pyruvate should contain isotope at positions 2 and 5 and, if formed from doubly labeled pyruvate, in positions 1 and 6 as well. Actually a significant excess of C^{13} was encountered only at positions 3 and 4. Thus a carboxylation of acetate to pyruvate cannot be responsible for the incorporation of acetate carbon into the glucose units of glycogen. Data pertaining to the formation of glycogen or intermediates of the citric acid cycle in the presence of labeled CO_2 do not permit any deductions as to the occurrence of acetate carboxylation because any labeled carbon entering by the latter reaction becomes indistinguishable from the carbon which is incorporated into the C_4 dicarboxylic acids by the primary carboxylation of pyruvate to oxalacetate.

$C_2 + C_2$ addition. There exist several observations which are consistent with Thunberg's suggestion (55) that succinate might arise by the linking of the methyl groups of two moles of acetate. The conversion of acetate to succinate by yeast with acetate as the sole nutrient was attributed by Wieland and Sonderhoff to the dehydrogenative coupling of acetate (153). In an analogous experiment with deuterio acetate Sonderhoff and Thomas (127) found high concentrations of deuterium in succinate and citrate and interpreted their results as evidence for reaction [10]. The proof for this contention would be conclusive only if the succinate contained close to four atoms of deuterium instead of the two atoms actually found. More than one pathway can account for the observed level of isotope in succinic acid. The same qualification applies to the findings on succinate formation from labeled carbon containing acetate by *Aerobacter indologenes* (108). The possibility that in both cases succinate arose by way of the tricarboxylic acid cycle cannot be excluded. At the present moment no experiments are available which establish the occurrence of reaction [10] in the conversion of acetate to succinate. Whenever succinate formation from acetate is demonstrable, succinate is not the sole product but α-ketoglutarate and tricarboxylic acids also accumulate. If acetate were metabolized according to equation [10] a mechanism should also be available for the subsequent conversion of succinate to ketoglutarate and citrate. The discovery of the carboxylation of α-ketoglutarate to oxalosuccinate by Ochoa (168) provides a pathway to the tricarboxylic acids, but the reversibility of the oxidative decarboxylation of ketoglutarate to succinate remains to be demonstrated.

$C_2 + C_3$ addition. The possibilities have been considered that acetic and pyruvic acids combine to form the five carbon acids α-ketoglutaric and aceto-pyruvic acids. As a result of the current prominence of the tricarboxylic acid cycle these two processes, which would lead directly to five carbon acids, have received diminishing attention. Krebs and Johnson (169) have suggested the formation of aceto-pyruvate according to the equation

$$CH_3CO\overline{OH + H}CH_2COCOOH \rightarrow CH_3COCH_2COCOOH$$
$$CH_3COCH_2COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COCH_2COOH + CO_2 + H_2O \quad [13]$$

as a conceivable intermediate for the conversion of pyruvate to acetoacetate in liver. The utilization of acetopyruvate by intact animals and in isolated tissues has been studied by Lehninger (170). The compound was found to be ketogenic. Since acetoacetate can be metabolized by way of the tricarboxylic acid cycle the reaction offers a chemically plausible scheme for the conversion of pyruvate to the oxidation level of acetate.

Acetopyruvate cannot be an obligatory intermediate in ketone body formation from fatty acids. Weinhouse et al. (76), in a study of the oxidation of labeled octanoic acid by liver, found an isotope distribution in acetoacetate which eliminated the participation of pyruvate as an essential component. It is however conceivable that ketone bodies can be formed by two independent synthetic processes, by condensation of two molecules of acetate and by combination of acetate with pyruvate.

α-Ketoglutarate is a key substance in the tricarboxylic acid cycle and a demonstrable intermediate whenever fatty acids or pyruvate are oxidized. A process leading to α-ketoglutarate by "end to end" condensation of acetate and pyruvate has been suggested by Krebs (171):

$$HOOCCH_2\boxed{H + H}CH_2COCOOH \rightarrow HOOCCH_2CH_2COCOOH \quad [14]$$

Weinhouse et al. have observed the formation of labeled α-ketoglutarate from isotopic acetate and normal oxaloacetate in rat kidney slices or mince, but found no evidence for the simultaneous formation of marked tricarboxylic acids (172). In their experiments non-isotopic citrate and cis-aconitate were added as carriers to facilitate the recovery of citrate at the end of the incubation period. The failure of acetate carbon to become incorporated into citrate in spite of the presence of aconitase, led the authors to the conclusion that citric acid as such was not in the pathway of acetate oxidation by kidney. These results would seem to suggest that α-ketoglutarate arose from acetate and pyruvate according to equation [14] without intervention of the tricarboxylic acids. However in this case the data exclude rigidly only the formation of citric acid itself. It does not appear to be feasible to isolate isocitric acid or cis-aconitate from such experiments. That the latter two tricarboxylic acids may have been intermediates becomes clear from kinetic data of Martius (173) and Krebs (174). These authors have shown that in systems containing the tricarboxylic acids and aconitase the conversion of cis-aconitate to isocitrate proceeds much more rapidly than to citrate. As the conversion of isocitrate to α-ketoglutarate is also a rapid reaction it is possible that in the experiments of Weinhouse et al. cis-aconitate or isocitrate were the initial reaction products but that the breakdown to α-ketoglutarate proceeded at a much faster rate than isomerization to citrate.

In a more recent investigation (175) the same authors report that after incubation of kidney with isotopic acetoacetate and normal oxaloacetate, without addition of non-isotopic carrier, citrate could be isolated directly and shown to

contain an isotope level which indicated that about two-thirds of the citrate arose from the isotopic acetoacetate. These recent experiments differed from earlier ones only in that acetoacetate instead of acetate was the substrate. In view of the fact that acetate is readily convertible to acetoacetate under these conditions it is not apparent why labeled citrate should not have formed in both instances.

In an analysis of the available data pertaining to the citric acid cycle, Wood (123) points out that α-ketoglutarate formation according to reaction [14] as opposed to its formation from tricarboxylic acids cannot be ruled out at the present. If reaction [14] were responsible for α-ketoglutarate formation rather than the oxidative decarboxylation of isocitric acid, then the formation of tricarboxylic acids would have to be ascribed to the carboxylation of α-ketoglutarate to oxalosuccinate and reduction of the latter to isocitrate. Experiments with labeled test substances and studies of isotope distribution in the α-ketoglutarate formed from either labeled acetate or acetoacetate have been incapable of providing an answer as to which reaction occurs. Labeled carbon will occupy the same positions whether α-ketoglutarate is formed by oxidative decarboxylation of isocitrate, directly from acetate and pyruvate or by carboxylation of succinate formed according to equation [10]. The condensation of oxaloacetate and acetate to a tricarboxylic acid does not as such involve an oxidation but can be formulated either as an addition if isocitrate is the primary product, or as a condensation with elimination of water if cis-aconitate is initially formed. It differs in this respect from reactions [10] and [14] in which the coupling process is oxidative. Experimentally it has been found that the formation of the intermediates of the citric acid cycle requires aerobiosis (176) unless a hydrogen acceptor such as oxaloacetate is present in excess (177). This may indicate the occurrence of oxidative condensations or, as is generally believed, oxygen may be necessary to effect the transformation of the reactants into activated forms or derivatives.

There is only scant information on the mechanisms by which carbon-carbon bonds are established biologically. It may be pointed out that in the few processes in which the chemical identity of the reacting molecules seems well established, e.g., in the formation of hexose from triose, or of acetoacetate from acetate, or in the carboxylation of pyruvate to oxaloacetate, at least one of the reacting groups is a carbonyl group. No instances are known of a formation of carbon to carbon bonds by elimination of hydrogen between methyl- or methylene groups.

$C_2 + C_4$ condensation. At present the ultimate oxidation of the split products of fatty acids can be explained most satisfactorily by assuming the intermediate formation of a tricarboxylic acid according to equation [11] and the subsequent breakdown of the condensation product by way of α-ketoglutaric acid and the C_4 dicarboxylic acids. Reaction [11] was originally proposed by Virtanen (178) to account for citrate formation from acetate in micro-organisms. The following evidence has been secured in support of the suggested rôle of the citric acid cycle for fat and acetate oxidation in various biological systems. Wieland and Sonderhoff (153) found succinate and citrate to accumulate as products of acetate

oxidation by yeast when acetate was the sole nutrient. Under these conditions, the yield of citrate is increased by the addition of oxaloacetate (179). Succinate and citrate formed from deuterioacetate contained 50 per cent and 46 per cent respectively of the deuterium concentration of acetate in stably bound form (127). Lynen has contributed important evidence to show that the formation of succinate from acetate in yeast involves the tricarboxylic acid cycle (157). He demonstrated that the malonate inhibition of acetate disappearance could be overcome by fumarate or oxalacetate and cites this finding as evidence for a requisite participation of the C_4 dicarboxylic acids in acetate oxidation. The observation that the rapid disappearance of acetic acid in yeast is preceded by an induction period was interpreted to show that a second component must accumulate before acetate can be metabolized at the maximum rate. A stimulatory effect of citrate, α-ketoglutarate and the C_4 dicarboxylic acids has been observed for the aerobic disappearance of acetoacetate in kidney homogenates by Buchanan et al. (180). Wieland and Rosenthal (176) obtained optimum yields of citrate in kidney brei when acetoacetate was oxidized in the presence of oxaloacetate. The identity of the reaction product with citric acid was established by direct isolation. Hunter and Leloir (177) have shown that two molecules of extra citric acid arise from each mole of acetoacetate. They have made the observation that in their experimental system, which consisted of the insoluble particles of kidney cortex homogenate, the simultaneous oxidation of α-ketoglutarate was essential for citrate formation. This oxidative process is believed to furnish the necessary energy for the conversion of acetoacetate to the intermediate which condenses with oxaloacetate. Lehninger (89) has found that the oxidation of octanoic acid in a preparation of washed rat liver cells yields acetoacetate quantitatively, but that in the presence of fumarate, citrate and α-ketoglutarate accumulate in significant quantities at the expense of acetoacetate. According to Breusch (181) an enzyme can be extracted from kidney, brain and skeletal muscle which catalyzes the anaerobic condensation of β-keto acids with oxaloacetate to citrate.

In addition to the balance studies a number of experiments have been carried out with isotopically labeled substances. Buchanan et al. (180) incubated kidney homogenates with $CH_3C^{13}OOH$ or acetoacetate marked by heavy carbon at both carboxyl and carbonyl positions. Non-isotopic ketoglutaric acid, fumarate or succinate were added as carriers; when re-isolated after incubation these acids were shown to contain C^{13} in the carboxyl positions. It was calculated that the γ-carboxyl of α-ketoglutarate contained almost ten times as much C^{13} as the carboxyl carbon adjacent to the carbonyl group. Essentially the same findings were obtained by Weinhouse et al. (172). The formation of labeled citrate from acetoacetate recently demonstrated by the same authors (175) has been mentioned above.

Intermediates of the citric acid cycle can be obtained from isolated tissues on incubation with a variety of substrates but since they are short-lived intermediates *in vivo* they are not obtainable as such in sufficient amounts from intact animals. It has therefore not been possible to secure direct evidence as to

whether the tricarboxylic acid cycle is concerned in fatty acid oxidation also in the intact animal. Feeding experiments have been carried out by Buchanan, Hastings and Nesbett (120) with acetic, propionic and butyric acids, all of which contained C^{11} in the carboxyl group. Glycogen isolated from such animals contained radioactive carbon in concentrations which in the case of acetate feeding were attributable to incorporation of CO_2, but were significantly higher with radioactive propionate and butyrate. Evidently the latter two acids had furnished carbon for glycogen synthesis by conversion to intermediates of carbohydrate metabolism. With the aid of degradation procedures which allow isotope determinations at individual carbon atoms of the glucose molecule (115), Wood and his collaborators have been able to show that the carboxyl carbons of acetic acid appear at positions 3 and 4 (121) and the methyl carbons at positions 1, 2, 5 and 6 of the glucose molecule (182). Thus, both carbon atoms of acetic acid are involved in glycogen formation. Butryric acid $CH_3C^{13}H_2CH_2C^{13}OOH$ afforded the same isotope distribution in glycogen as $CH_3C^{13}OOH$ (182). From these findings the conclusion can be drawn that there exists a pathway for the conversion of the two carbon split products of fatty acids into the glucose units of glycogen. In this process each carbon atom of glucose can originate from fatty acid carbon.

Glutamic and aspartic acids are believed to be in biological equilibrium with α-ketoglutarate and oxaloacetic acid. Any incorporation of marked fatty acid carbon into intermediates of the tricarboxylic acid cycle should therefore become manifest by the formation of labeled aspartic and glutamic acids. The two amino acids have been isolated by Rittenberg and Bloch from the tissue proteins of mice which had received $CH_3C^{13}OOH$, and were shown to contain significant concentrations of C^{13} (183).

While the evidence which has accumulated in favor of the tricarboxylic acid cycle is impressive it should be emphasized that this scheme does not offer a unique explanation for the formation of tri- and dicarboxylic acids as products of fatty acid oxidation. The following points of the cycle need further clarification: 1, the identity of the intermediate of fatty acid and pyruvate metabolism which condenses with oxaloacetate; 2, the nature of the initial condensation product; 3, the existence of one or more mechanisms for the oxidation of acetate, acetoacetate and pyruvate respectively.

Although the stimulatory effect of the C_4 dicarboxylic acids on citrate formation is exhibited not only by oxaloacetate but also by succinate, malate and fumarate, it is reasonable to asume that the effect is attributable in all cases to oxaloacetate. The nature of the acetyl compound which participates in the condensation process is less evident. In some systems intermediates of the cycle are formed from acetate as well as from acetoacetate, while in other situations the two substances cannot be employed interchangeably. In balance studies with kidney preparations (176, 177) and in the experiments of Lehninger (66) with a heart muscle preparation only acetoacetate was an effective precursor of citrate. The failure of acetate to react in kidney preparations is unexpected because acetate is readily converted to acetoacetate in this organ. On the other hand,

Buchanan et al. (180) found that labeled acetate was as effective as acetoacetate in forming labeled α-ketoglutarate in kidney homogenate. It should be reemphasized in this connection that with the aid of the tracer technique a conversion can be demonstrated even if there is no net change in the concentration of the reaction product, e.g., citrate or α-ketoglutarate. This is important in systems in which the product is metabolized further at a rapid rate.

In general, acetoacetate appears to be the more reactive molecule with regard to the condensation reaction. On the basis of their data Wieland and Rosenthal (176) postulated that acetoacetate itself condensed to yield the hypothetical procitric acids, citroylacetic or acetylcitric acids. Weinhouse et al. were able to isolate C^{13} containing citrate from kidney homogenate when acetoacetate was the substrate (175), while in earlier experiments under similar conditions, labeled acetate yielded isotopic α-ketoglutarate but not citrate (172). The preferential utilization of acetoacetate might be taken to indicate that acetate enters the tricarboxylic acid cycle by way of acetoacetate. However, several situations exist for which this explanation does not hold. Citrate is a product of acetate oxidation in yeast, but as far as is known, the conversion of acetate to acetoacetate does not occur in this organism. Medes et al. have shown that acetate undergoes complete oxidation in heart muscle but that acetoacetate is not an intermediate (86). In kidney also a portion of acetate was oxidized by a route which did not pass through the acetoacetate stage. The data allow for two possible explanations: 1, acetate and acetoacetate enter the tricarboxylic acid cycle by a different process, and 2, a reactive acetyl compound is formed from both acetate and acetoacetate before condensation occurs. In the utilization of acetoacetate a preliminary splitting to acetic acid can be excluded as an intermediate step. Buchanan et al. (184) added normal acetate and isotopic acetoacetate to an extract of rabbit kidney and found that the acetic acid recovered from the mixture after incubation contained appreciably less isotope than α-ketoglutarate or succinate. From energy considerations it is to be expected that an energy rich acetyl can arise far more readily from acetoacetate than from acetate. The inability of acetate to substitute for acetoacetate may be ascribed to an impairment of the enzyme system responsible for the conversion of acetate to the metabolically active form. Although acetoacetate is an efficient source of citrate in various tissue systems, it is highly doubtful that it is an obligatory intermediate in tricarboxylic acid formation from fatty acids. In the experiments of Lehninger with washed liver cells, acetoacetate, unlike octanoic acid, was incapable of yielding citrate (89). The preparation apparently had lost the property of converting acetoacetate into the active two carbon fragment. The reasons which led to the belief that in the intact animal acetoacetate is not an intermediate of fatty acid oxidation have been discussed above.

Since the experimental conditions employed by various investigators for the study of the same problem vary widely, particularly with respect to methods of tissue preparations, it is not surprising that such experiments have often yielded contradictory results and that it is not possible to fit all available data into a single scheme. It is evident that with increasing impairment of the cellular

organization the enzymatic complement of the biological system becomes increasingly deficient and hence the probability will diminish that a complex series of reactions can take place. Processes which have high energy demands and must be coupled with energy yielding reactions such as the conversion of acetate to an active acetyl are less likely to occur in homogenates or tissue extracts than in systems in which the cell structure is preserved.

Lipmann has shown that by the addition of adenosine triphosphate acetate can be activated to acetylate sulfanilamide anaerobically in pigeon liver extracts (2). The expectations, based on this finding, that the active C_2 compound which is involved in the tricarboxylic acid cycle may be identical with acetyl phosphate, have not been fulfilled. Under conditions in which citrate formation can be demonstrated to occur with acetoacetate but not from acetate, acetylphosphate is also ineffective (184, 177, 89). In order to determine the identity of the active two carbon compound, Lehninger (89) has also tested the effect of glycolate, glyoxalate, oxalate, acetaldehyde, ethanol, acetamide and glycine. None of the compounds yielded extra citrate under conditions which afforded citrate from octanoic acid.

Since cis-aconitate, isocitrate and citrate are biologically interconvertible, the identity of the tricarboxylic acid initially formed in the citric acid cycle has remained in doubt. In the presence of aconitase the system will contain all three acids irrespective of the nature of the primary condensation product (173, 185). The cycle as originally proposed by Krebs and Johnson (185), in which citric acid was the initial condensation product, had to be modified when it was found by Evans and Slotin (186) and by Wood et al. (187) that the α-ketoglutarate formed in pigeon liver homogenate from labelled CO_2 and pyruvate contained labeled carbon only in the carboxyl group adjacent to the carbonyl group, and that when malonate was present, the succinate formed contained little C^{13} (186). The tricarboxylic acids could therefore not have originated from a symmetrical citric acid. An unsymmetrical molecule must also have been the precursor of the dicarboxylic acids which Buchanan et al. (180) and Weinhouse et al. (175) obtained as the reaction product of labeled acetoacetate or acetate, and oxaloacetate. In the α ketoglutarate obtained from the latter experiments only one-sixth and one-fourth respectively of the total isotope was present in the carboxyl adjacent to the keto group; it should have contained half of the total, if a symmetrical tricarboxylic acid had been the precursor. Likewise, in glutamic acid isolated from the proteins of animals which had received carboxyl labeled acetic acid the heavy carbon was not evenly distributed between the two carboxyl groups (183). The C^{13} content of the carboxyl adjacent to the amino-group accounted for only one-fourth of the total, whereas it should have contained half of the total if the α-ketoglutarate arose from a symmetrical precursor. Thus, in the intact animal also, the α-ketoglutarate formed in the process of acetate oxidation is derived from an intermediate in which the orientation of the labeled carbon is preserved. The much higher isotope content of glutamic than of aspartic acid isolated from this experiment suggests that only a small fraction of the isotope incorporated into the glutamic acid was contributed by CO_2 assimila-

tion. The carbon chain of aspartic acid is believed to be furnished by oxalacetate. If isotope had entered exclusively in the form of CO_2 by the carboxylation of pyruvate to oxalacetate, then aspartate should contain not less but as much or more heavy carbon than the α-ketoglutaric acid which is subsequently formed from oxalacetate by way of a tricarboxylic acid.

The results obtained with labeled substances eliminate a symmetrical molecule as the initial condensation product, but do not indicate which tricarboxylic acid is involved. In addition to isocitrate and cis-aconitate, the two acids which according to present views are the most likely intermediates, oxalosuccinate has become a possible intermediate since Ochoa (168) has demonstrated reversibility of the isocitrate-oxalosuccinate conversion. Citric acid has a symmetrical carbon chain but the possibility still exists that a citric acid which is substituted at one of the two primary carboxyl groups lies on the main path of the cycle. The suggestion of Wieland and Rosenthal (176) that the citric acid formed in kidney from acetoacetate might have arisen secondarily from a procitric acid containing eight carbon atoms has been mentioned. Lynen (188) finds the formation of citrate from acetate and oxalacetate by yeast to be promoted by the simultaneous oxidation of succinaldehyde and believes that the function of this reaction is to convert either one of the components into a more reactive derivative. If the condensation takes place with an acetyl compound CH_3 COR, a substituted unsymmetrical citrate molecule may result, which could be converted to cis-aconitate without elimination of the substituent R. Lynen has pointed out that the data of Sonderhoff and Thomas (127) on the formation of labeled citrate and succinate in yeast, also rule out a symmetrical citric acid. Succinate formed from trideuterioacetate by way of a symmetrical tricarboxylic acid could not have contained more than one atom of deuterium, but was actually found to contain two atoms. The suggestion that an unsymmetrically substituted citric acid derivative is initially formed would also account for the unequal distribution of isotopic carbon in α-ketoglutarate formed from labeled acetate or acetoacetate. The question concerning the identity of the initial reaction product might be clarified materially if it were possible to establish whether citrate formation occurs only in systems which contain the enzyme aconitase. According to Krebs (162) this enzyme is absent from yeast. In this case the citric acid arising in the course of acetate oxidation by this organism could not have been formed secondarily from isocitrate or cis-aconitate but must have been the primary condensation product.

A discussion of the rôle of the citric acid cycle in carbohydrate metabolism is beyond the scope of this review, but it may be pointed out that no evidence exists to support the contention that the tricarboxylic acid formed in the oxidative metabolism of pyruvate is identical with that arising from fatty acid oxidation. The finding of Martius (189) that oxalocitramalate, the condensation product of pyruvate and oxalacetate, is not attacked by kidney tissue is hardly sufficient evidence to rule out the occurrence of intermediate C_7 tricarboxylic acids. As the experience with yeast has shown (190, 157), the lack of metabolic activity of a substance added to a biological system may be apparent only and

may be caused by inability of the substance to penetrate cell walls. This is particularly true for polycarboxylic acids. Furthermore, there are other C_7 acids which could conceivably arise from pyruvate and oxaloacetate and which have not been tested biologically (formylcitric, oxalocitraconic acids).

The conclusion that the tricarboxylic acid cycle provides an important mechanism for the complete oxidation of fatty acids in the intact animal is based on evidence of indirect nature. The appearance of isotopic carbon in the glycogen of animals fed labeled fatty acids and in the dicarboxylic amino acids after feeding of acetate is not by itself indicative of the mechanism responsible, but is most reasonably ascribed to pathways which lead to the intermediary formation of tricarboxylic acids. It is not possible at present to determine what portion of the fatty acids is oxidized in the intact animal by this route. From the high isotope level in glycogen in the experiments of Buchanan et al. (120) and of Lorber et al. (121) it is clear however that a significant fraction of acetate and butyrate respectively was metabolized by way of the tricarboxylic acid cycle. In these experiments with starved animals, ordinary glucose was given simultaneously with the labeled fatty acids in order to insure glycogen deposition. The labeled glycogen arising from the labeled fatty acids was therefore diluted by glycogen formed directly from dietary glucose.

The finding of Wood et al. (182) that the same isotope distribution in glucose obtains with butyrate labeled at carbon atoms 1 and 3 as with carboxyl labeled acetate demonstrates the breakdown of butyrate to two acetyl groups and furnishes additional evidence for the view that fatty acids are degraded by successive removal of two carbon units. These data are not in agreement with the suggestion of Blixenkrone-Moeller (191) that butyrate might be oxidized to succinate directly by ω oxidation.

The results obtained during the past few years have not only provided the basis for a reasonable scheme of the total oxidation of fatty acids but have also clarified the controversial relationship of fat and carbohydrate metabolism. From the findings discussed above it may be concluded that fatty acids are convertible to carbohydrate in a restricted sense. The split products of fatty acid oxidation contribute to carbohydrate synthesis but the fatty acids by themselves are incapable of causing an increased formation of glucose or glycogen because the mechanism by which the fatty acid carbons find their way into glucose requires the participation of carbohydrate itself. This explains why in the starved or diabetic animal fatty acids and acetate do not show a glycogenic effect.

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BIOLOGICAL AND MEDICAL APPLICATIONS OF ELECTROPHORESIS

JOHN A. LUETSCHER, JR.

Department of Medicine, Johns Hopkins University and Hospital, Baltimore, Md.

The need of an effective method for the separation and analysis of mixed proteins has been uniquely met by recent improvements in the electrophoretic method. The ordinary principles of qualitative analysis are inadequate to deal with proteins, whose components are similar and whose characteristics are determined less by their composition than by their structure. Furthermore, proteins may be easily modified and their distinctive properties may be destroyed by the chemical treatment necessary for their separation. The physical methods are better fitted for the task. A distinctive structural characteristic of the protein is used in the separation, and the forces applied are extraordinarily harmless. The amount of material necessary for physical analysis is small. Ultracentrifugation (329) and electrophoresis share these properties and yield valuable, independent information on the characteristics and composition of proteins.

Technique. The movement of protein ions in an electric field has emerged during the last decade as an important biochemical method (45, 165, 170, 186, 214, 323, 324, 328, 338, 355, 359). Earlier studies were limited by technical difficulties and the present usefulness of the electrophoretic method has developed largely from improvements in technique. Ten years ago, Tiselius described the apparatus which gave speed and precision to the moving boundary method and made possible the quantitative study of complex proteins (348). Longsworth, Philpot, and Svensson adapted the optical system to simplify the quantitative analysis of mixtures (116, 131, 163, 164, 165, 170, 173, 213, 250, 330, 331, 338). These technical improvements opened new fields of investigation, which have been widely explored and described in a literature so extensive as to be difficult to bring together in a review of reasonable length. This review is limited almost entirely to the work of the last decade. The reader will doubtless find applications to his own field of interest which can be fully appreciated only after study of original work in that field.

In the "moving-boundary" method of electrophoresis, a sharp "boundary" between a protein solution and its solvent is formed and observed as the protein moves in an electric field. If the protein is homogeneous in mobility, the boundary remains sharp and single. If there are different species of protein in the solution, they may move with different speeds, so that the original, sharp, protein-solvent boundary becomes wider and may separate into several boundaries, each representing the end of a column of protein of different mobility. The number and position of the boundaries are observed by means of the change in refractive index produced by the change in protein concentration. If the specific refractive increment of each protein is known, the concentration of the components of a mixture may be measured. Under suitable conditions, the

slowest and fastest components may be separated from the mixture and recovered in nearly pure form.

Electrophoretic study of insoluble proteins can be made by the microscopic observation of the motion of particles. Inert particles coated with protein generally move with the same mobility as the dissolved protein (221, 222, 223, 300, 307). The surface of living cells may be studied by similar observations. Abramson, Moyer, and Gorin (10) have reviewed the earlier micro-electrophoretic studies and included an account of their interesting studies on cells. Electro-osmosis and membrane potentials are considered to be corollaries of electrophoretic migration.

Observation of purified proteins has brought out the dependence of electrophoretic mobility on the pH and ionic strength of the solution, and curves of mobility at various hydrogen-ion concentrations are closely related to the acid-base titration curves of the protein or other amphoteric substance (4, 10, 54, 322, 338, 360). The mobility of protein is affected not only by the quantity of ions present but also by the types of ion in the solvent (16, 338, 349). Since the relative mobilities of the components of a mixture determine the completeness of electrophoretic separation, the choice of buffer is of critical importance. Furthermore, the boundary anomalies and the corresponding uncertainty of analytical accuracy may be reduced in proper buffers (66, 165, 167, 170, 336, 338). The avoidance of convection requires accurate adjustment to the temperature of maximum density of the solution studied (12, 165, 207, 211).

Electrophoretic Observations and Applications. The simplest type of observation which can be made by electrophoresis is the enumeration of boundaries. The component protein fractions may be further described according to their mobility under one or more conditions of pH and ionic strength. Tiselius and Stenhagen described the electrophoretic fractions of the plasma proteins in this way, identifying albumin, fibrinogen, and the three globulin fractions which were named alpha, beta, and gamma in the order of their mobilities (320, 348, 349, 350).

Electrophoretic homogeneity is sometimes interpreted as chemical homogeneity without adequate proof or reservation. There are many other important criteria of the purity of proteins, including chemical analysis, crystallinity, ultracentrifugal analysis, and, perhaps, most important, constant solubility (302). Biological activity may be used as a crude measure when present. These methods are unfortunately contradictory in some cases. The weight which is placed on the electrophoretic measurements should depend on the completeness of examination under wide variations of pH, ionic strength, and buffer salts, with a protein concentration adequate to detect small amounts of an impurity. In certain cases, it is easy to decide the relative value of electrophoresis. Crystals of serum albumin, egg albumin, or hemoglobin, studied by electrophoresis, may show recognizable contaminants from the native mixture (145, 168, 187, 216). A pure preparation may become electrophoretically impure on standing (216, 304). On the other hand, insulin appears the same to electrophoresis, whether the preparation is crude or purified (100), and solubility has proved a

more sensitive criterion than electrophoresis in the study of pepsin (107) and chymotrypsinogen (28). Ultracentrifugation may yield simpler patterns than electrophoresis, as in normal serum globulin (242, 329) and enzymatic digests (61, 248, 362), or more complex patterns than electrophoresis, as in the abnormal serum globulins of multiple myeloma (130, 196, 242). Electrophoretic homogeneity is obviously a very limited measure of purity when the preparation has been made by electrophoretic separation.

The most fruitful use of this type of observation has been in the guidance of chemical separation of proteins from natural sources. The results have been equally important to the student of electrophoresis, since they have identified the chemical and biological nature of the electrophoretic components.

In extensive studies of plasma proteins, E. J. Cohn and his associates have used the electrophoretic method as a control in fractionation (43, 44, 45, 46), obtaining not only purified fractions of biological and medical value, but also a vast store of information correlating chemical and biological properties with electrophoretic behavior. Electrophoretic control of classical and newer methods of plasma fractionation has found general acceptance (61, 62, 63, 93, 119, 332, 349, 350, 381). This co-ordinated approach has been less often applied to the proteins altered by disease in order to elucidate the changes inadequately described by electrophoresis alone. Longsworth (169) demonstrated the increased lipoid content of the beta-globulin in nephrosis and in obstructive jaundice. Recently, Seibert (290) has correlated the increased polysaccharide content of blood with the increase in alpha-2-globulin seen in pneumonia, tuberculosis, and certain forms of cancer.

The striking changes which occur in the electrophoretic pattern of serum during immunization and after reaction with the specific antigen give a clear picture of the appearance and removal of antibody. Such studies have been equally important in the understanding of the electrophoretic pattern and in establishing the relationship of antibody to the plasma proteins. The early studies of Tiselius and Kabat showed that circulating antibodies appeared in the gamma-globulin fraction of rabbit plasma (350, 353, 358) and this has been abundantly confirmed in many species (52, 53, 57, 73, 132, 206, 209, 231, 232, 274, 289, 341, 345, 365, 366, 369, 382, 383). Even the "normal" adult man bears the scars of old battles with disease in his gamma-globulin (63, 71). The increased globulin after immunization is not necessarily identical with normal gamma-globulin (63, 104, 174, 353, 358, 365, 366, 368), nor is the increase in the gamma fraction entirely active antibody (270, 368, 371, 382). Less often, some antibody has been found in other globulin fractions (71, 73, 132, 239, 286). The electrophoretic method has been useful in studying the chemical purification of immune globulin, but has found only a minor place in the preparation of highly purified antibody (335), since other methods have proved to be more specific and effective.

Some information on the origin of antibody has been contributed by White and Dougherty (69, 378, 379), who have shown that several proteins resembling those of serum may be formed in lymph nodes. These proteins are released from

the lymph nodes under pituitary adrenocortical stimulation at the same time that antibody increases in the serum. Since the adrenocorticotrophic hormone may also increase serum albumin (160), the ensuing relative changes in serum proteins may vary and can not be interpreted in the absence of measurements of plasma volume. It seems quite clear, however, that several proteins, including antibody, may be increased in the circulating blood after pituitary adrenocortical stimulation. The rôle of the lymph node in the production of gamma-globulin has been corroborated by the demonstration in lymph nodes of a protein antigenically identical with the gamma-globulin of serum (127).

The purification of proteins with hormonal and enzymatic activity has been aided by electrophoresis as a control and, in some cases, as a separative tool. Theorell combined the chemical and electrophoretic methods in his purification of the yellow enzyme (128, 343) and later of cytochrome C (344). Preparations of pepsin (106, 107, 352), chymotrypsinogen (28), phosphorylase (89, 374), zymohexase (310), 1-amino-oxidase (90), carboxylase (202), ribonuclease (269), mucopolysaccharase (98, 99), and carbonic anhydrase (247, 311) have been examined. Enzymatic digestion of protein has been followed by electrophoresis (61, 248, 249, 356, 362, 363, 370), but ultracentrifugation is generally a more sensitive measure. The preparations of the protein hormones of the pituitary have been guided by electrophoresis (38, 39, 151, 152, 153, 154, 156, 157, 159, 228, 277, 299, 304, 376, 377). In the case of insulin, however, the method has not proved applicable (100). Highly purified thyroglobulin is homogeneous in its migration (103).

Many proteins of cells and their secretions (35, 36, 58, 59, 70, 91, 101, 114, 115, 118, 122, 133, 155, 177, 200, 201, 217, 225, 258, 265, 315, 325, 327, 354, 357, 380, 387, 388) have been subjected to electrophoresis, generally to estimate the purity or complexity of a preparation or to separate an active material, such as the hematopoietic factor of the liver (375) or the thromboplastin of lung (41). Other materials, such as hemoglobin or egg albumin (11, 13, 18, 29, 54, 83, 168, 240, 263) have been extensively studied because of their availability, ready crystallization, and the wealth of comparative physico-chemical information, as well as for the interest in their biological properties (27). Bacterial antigens and toxins (1, 47, 82, 125, 126, 142, 162, 192), as well as active preparations of virus (24, 25, 72, 79, 146, 197, 198, 204, 229, 256, 257, 271, 293, 300, 303, 307, 308) have been studied. Plant extracts of many types show complex patterns which can usually be related to their chemically separated components (15, 75, 76, 77, 117, 264, 273, 280).

A number of interesting investigations deal with cell proteins which commonly give rise to hypersensitivity in man. Seibert's purification of tuberculin proteins, guided by electrophoresis, has led to a standardized testing material of medical importance (92, 282, 283, 284, 285). Abramson, Moore, and Gettner have separated the proteins of hay-fever producing pollens by electrophoresis and have identified skin-reactive fractions of low molecular weight, which may account for the unusual sensitizing ability of these pollens (6, 7, 8, 9). Chemical methods of separation of pollen fractions have not been so effective under electro-

phoretic scrutiny (233). Electrophoresis has found a place in the separation of the allergenic protein of cottonseed (316, 317).

Intact living cells move in the electric field. The influence of the solvent on this motion may reveal properties of cell surfaces, which determine the mobility (2, 10, 80, 105, 319). Intact red blood cells possess distinctive surface characteristics which are little altered by suspension in protein solutions (5) and surprisingly unaffected by damaging agents (14). The ghosts of hemolyzed red blood cells migrate with the same speed as the intact cell (3) and a component of similar mobility has been identified in extracts of the cells (326). Spermatozoa show variations in isoelectric point and mobility, which allow separation and perhaps some degree of sex differentiation (305, 306). Bacteria migrate in the electric field, and some biological characteristics may be correlated with mobility (26, 33, 78, 161, 236, 319, 372). Similar studies of protozoa have been made (193, 291, 305).

Preparative Electrophoresis. It is often desirable to recover one or more of the fractions separated by electrophoresis for chemical characterization or for study of biological activity. Since separation and recovery are most successful when the mobilities of the components are distinctly different, the conditions should be chosen with great care. In any single separation, only the fastest and slowest components can be obtained in pure form. Fractions of intermediate speed can be obtained by two or more steps, first isolating two faster components, for example, and then separating them in a second operation. Given a mixture which can theoretically be separated, the chief problems are concerned with technique, purity, and yield. In general, techniques which give fractions of the highest purity also yield very little material (337) and the collection of large amounts of material is very tedious. The conventional U-tube design of Theorell (342) and Tiselius (348, 351) includes apparatus of varying size and with varying numbers of compartments which can be closed off at the end of the separation. Macheboeuf (184) described an ingenious method of withdrawing samples without disassembling the apparatus. Svensson (333, 334, 338) uses a similar technique in his apparatus, which is arranged to allow almost continuous operation. There are other quite different principles of separation which have not yet reached the technical perfection of the conventional apparatus (337, 338), but which have great promise in suitable circumstances. "Electrophoresis-convection" causes the concentration of protein against a semi-permeable membrane (94, 135, 343). Stationary electrolysis separates proteins of different isoelectric points (97, 361). Flowing, thin layers of protein have been used to facilitate separation (251). Packchanian described a large horizontal apparatus suitable for separation of living organisms (238).

Analysis of Plasma Proteins. The quantitative analysis of the component proteins of mixtures has had its widest application in the analysis of plasma and of certain related fluids. The original nomenclature of Tiselius is still in general use for the serum globulins, which are described as alpha, beta and gamma in the order of their mobilities in alkaline solutions. Certain subdivisions have appeared: two alpha and two beta globulin fractions are often further identified by

the subscripts 1 and 2. The complexity of the fibrinogen fraction is indicated by the appreciable protein of the same mobility remaining after clotting (385), which has been isolated and found to resemble gamma-globulin (63). The wide, reversible spreading of the gamma boundary shows its heterogeneity (116, 121, 294, 295). Albumin separates into two components on prolonged electrophoresis (22, 113), especially just above pH 4 (178, 296) or in the presence of certain anions (16, 336).

In the "normal" man and in certain animals, the electrophoretic fractions have acquired more or less substance of chemical and physical characterization. Such information confirms the complex nature of the albumin and globulin fractions (43, 44, 45, 46, 112, 199, 296, 332), as well as the presence of biologically active proteins in amounts too small to recognize in the pattern of the native proteins (45, 237, 252, 255, 281, 321). The association of carbohydrates and lipoids with the blood proteins is very intimate (21, 22, 34, 185, 350, 386) and these substances affect both the refractive increment and mobility of the proteins to which they are bound, chiefly the alpha- and beta-globulins (21, 23, 140, 143, 249, 385, 386).

When the proportions of the serum proteins are altered by disease, there may be qualitative changes in the fractions and not simply more or less of the normal proteins. In the study of such changes, it is important to employ suitable physical, chemical and biological techniques to correlate the electrophoretic patterns with more definitive alterations in the proteins. Many current reports are descriptive, a natural stage in the collection of information. It is to be hoped that the interest so aroused will stimulate the collection of more fundamental information on the altered proteins, so that these fractions, now only shadows on the electrophoretic diagrams, may be better defined.

The electrophoretic analysis of human plasma has become rigidly standardized in many laboratories, since not only the quantitative distribution of proteins but even the identity of fractions may vary somewhat with changes in the buffer (16, 50, 136, 170, 217, 245, 336). The human alpha-1-"globulin" of the most generally used barbiturate buffer is included in the "albumin" fraction in phosphate buffer, while the alpha-2 and beta globulins are often more clearly separated in phosphate buffer. Two useful buffers about which the most information has accumulated are sodium diethyl barbiturate of ionic strength 0.1 and pH 8.6, and sodium or potassium phosphate of ionic strength 0.2 and pH 7.7. Since too high a protein concentration affects the analytical results in buffer of low ionic strength (16, 65, 345, 336), the design of the optical system must conform to certain standards in order to obtain the best results. This total standardization leads to a single end, the comparability of human plasma patterns. For purposes other than the standard quantitative analysis of human serum or plasma, the investigator can adapt these conditions to produce optimum separation of the components which he wishes to study. The enormous flexibility of the electrophoretic technique should not be impaired by too rigid conventions, nor should sight be lost of the artificiality of any such single set of conditions.

The interpretation of the shadow diagrams must be guarded in certain respects. Interaction between proteins and other proteins (35, 47, 139, 141, 168, 217, 354,

357, 359) or smaller ions (16, 17, 171, 183, 205, 230, 259, 261, 322) may produce anomalous results. The changes occurring after prolonged standing (16, 143, 176, 187, 216, 279), freezing, drying, or denaturation (49, 55, 64, 102, 138, 140, 141, 188, 248, 262, 309, 350, 367) are defined for some proteins and mixtures. Moreover, the usual analytical method assumes the uniform refractive increment of components. These include lipoids and carbohydrates bound to serum proteins, and conversion factors are necessary in order to apply the data to nitrogen content (16, 48). The normal conversion factors may change under pathological conditions (16, 169, 386).

In spite of these criticisms, the electrophoretic method gives an accurate, reproducible, and distinctive description of the plasma proteins, which is not attained by any other available method (16, 50, 338, 354). It was long ago recognized that the "salting-out" of proteins yields only a limited analytical approach (37, 65, 88, 179, 219, 341, 349, 350, 371, 385). Original globulin precipitates studied by electrophoresis are complex mixtures, and the separation of albumin from globulin requires several recrystallizations. Alcohols yield better separations (44, 46, 254, 332), but require exact control of conditions.

The normal pattern of plasma has been established not only for man (16, 65, 88, 95, 129, 143, 144, 166, 179, 210, 235, 245, 254, 272, 279, 301, 330, 341), but for a number of animals (60, 136, 150, 158, 218, 275, 297, 336, 338). The analyses vary somewhat with different buffers (218, 336, 338) but indicate a striking species variation in the number and distribution of protein components. The normal electrophoretic pattern of human plasma is comparatively simple, since the separation of albumin, fibrinogen, and the three globulins is quite complete. The nomenclature of Tiselius for these well-defined fractions becomes quite artificial when applied to very complex animal sera, such as those of the sheep, cat, and guinea pig, in which as many as ten peaks may appear (60). Arbitrary definition is certain to lead to confusion, and a surer method of description of such complex sera is by mobility under defined conditions of buffer ions, ionic strength, pH, and protein concentration. The buffers in which many of these sera have been examined were chosen by systematic trial for optimal separation of human serum. More satisfactory buffers may well be found for individual animal sera.

From reported analyses it appears that ungulates tend to have a lower proportion of albumin in plasma than rodents and primates (60, 218, 338). The horse has the lowest recorded proportion of albumin. The plasma proteins of the rhesus monkey have a pattern closely resembling the human pattern. Birds show a rather characteristic mass of slower-moving globulins; and the "beta-globulin disturbance," fibrinogen, and "gamma-globulin" are poorly separated. The hen and cock may show differences in serum proteins, while in mammals the serum proteins show little or no sex differentiation. The carp has a pattern quite different from the higher animals (60, 218). Animals show considerable variations of protein distribution between individuals and breeds in addition to the species differences (218, 297, 298, 338).

In developing chick and pig embryos, the plasma undergoes a gradual differen-

tiation from a simple to a more complex pattern (220). The carbohydrate content falls during the period of development, while the nitrogen content rises. Albumin appears later than several components with the mobility of globulin (220). The blood proteins of the ruminant fetus are apparently quite different from those of the human or rodent fetus. The human fetus at birth has only a slightly lower albumin and higher gamma globulin than the adult man (144, 172, 279). The new-born calf, however, has almost no gamma-globulin and little beta-globulin until colostrum is ingested, when there is a remarkable increase in these globulins and the associated antibodies (120, 274). Pedersen (241, 242) has found a characteristic serum globulin which comprises a large part of the globulin of the ruminant fetus, but is found only in small amounts in the fetus of man or rabbit. These differences are probably related to the less permeable placenta of the ruminant. After weaning, the young dog may develop a lower albumin, fibrinogen, and gamma-globulin, and a higher alpha- and beta-globulin than the adult dog (150).

When quantitative analysis by electrophoresis was applied to human plasma in disease, surveys indicated such distinctive patterns as to arouse hopes for a wide application in medical diagnosis. Characteristic electrophoretic patterns have indeed become recognized as virtually diagnostic of certain diseases, such as nephrosis and some types of myelomatosis. More often, however, the picture has proved to be characteristic not of the specific disease but of the host's reaction to infection or injury. The various changes are frequently proportional to the severity of the physiological disturbance and may vary with the duration or stage of the disease, with nutritional factors, with loss of plasma protein, and with the involvement of certain organs, such as the liver (88, 182, 218, 290).

The common denominator of almost every pathological state is a relative or absolute decrease in the serum albumin. The electrophoretic technique demonstrates this change with greater sensitivity than the salting-out methods. The reduction of serum albumin is often associated with two factors common to many diseases,—a deficiency of protein and a general reaction of the body to injury and infection (31, 81, 190, 244). When the concentration of serum albumin is seriously reduced, the concentration is stabilized by osmotic factors and by the removal of transfused albumin from the circulation (181). Electrophoretically homogeneous fractions, such as concentrated human serum albumin, can be effectively used to study such transfers of protein (181, 346, 347).

The effects of malnutrition on the electrophoretic pattern of human plasma have not been adequately described. To be sure, there seem to be as many possible types of malnutrition as types of disease, but very little fundamental information of any sort is available. Keys (134) found normal electrophoretic patterns in volunteers who showed great weight loss and edema on a diet characteristic of the recent European famine. Patients with a history of prolonged alcoholism and multiple dietary deficiencies have developed intractable hypoproteinemia with a reduction of both serum albumin and globulin (182), but similar changes have been observed in patients without obvious dietary deficien-

cies (328). In one case, a very low concentration of gamma-globulin has been observed to increase after an adequate intake of protein (137).

The plasma of dogs on a diet deficient in protein with or without plasmapheresis shows a considerable reduction in serum albumin (37, 384, 385). The globulins are apparently increased, due to associated lipids, but the globulin-nitrogen of plasma is not increased (385, 386). The regeneration of albumin after depletion by diet and plasmapheresis takes a matter of weeks and is still incomplete when the total plasma protein has returned to normal levels (384, 385). Pigs on a tryptophane-deficient diet may develop a marked reduction in serum albumin and in several globulin fractions (30).

Severe bodily injury is followed by striking changes in the albumin and alpha-globulin fractions of the plasma. Burns, freezing, skin irritants, certain poisons, hemorrhage, fractures of bones, and myocardial infarction are followed by an increased alpha-globulin concentration (31, 81, 244, 301). The reduction of albumin may be a measure of the severity of injury and occurs earlier than the increase in globulin. The close resemblance to the changes observed in acute, febrile infections is unmistakable and suggests that this reaction of the proteins to injury is related to adrenal stimulation and the protein-catabolic reaction, which appear in similar circumstances.

The plasma globulins respond to infection in two distinctive patterns. In the febrile stage of an acute infection there is usually an increase of alpha-globulin to several times its normal concentration. The usual reduction in serum albumin makes the proportional rise of the alpha-globulin even more striking. This change has been observed in pneumonia (166, 180), tonsillitis (166, 190), scarlet fever (68), rheumatic fever (68, 166, 190), peritonitis (166), and infectious mononucleosis (42). An exception to the general association of acute febrile illness with an increased alpha-globulin occurs in vivax malaria (67, 96). The increased alpha-globulin may be related to the "C-reactive protein" which appears in human serum in acute infections and which is found in the alpha-globulin fraction (243).

A later change in serum globulin following virtually all types of infection is an increased concentration of the gamma-fraction (42, 53, 67, 68, 96, 166, 180, 190, 191, 208, 231, 272, 288, 290). The time-relationship of these changes has been clearly demonstrated in a continuous study of the plasma during the course of scarlet fever and rheumatic fever (68). The increase in gamma-globulin is probably correlated with the production of antibodies. The new component may not have a mobility identical with the normal fraction (63). In kala-azar, a fraction of unusually slow mobility appears in large amounts (51).

In chronic infections of many types, there is a tendency for all globulins to become increased in a manner reminiscent of "hyperimmune" sera of animals (366). In many infections, fibrinogen is increased at an early stage along with alpha globulin and may remain elevated if the disease persists (190). A recent survey of many cases of tuberculosis describes changes in the serum globulins varying with the stage of the disease (290). In minimal tuberculosis, gamma globulin is elevated above the normal level. Moderately advanced cases show

parallel increases in the alpha-2-globulin fraction and in the closely associated polysaccharide of the serum. In far advanced tuberculosis, all globulin fractions are increased (287, 288, 290). The total protein of the serum usually remains normal in spite of a progressive fall in the albumin concentration (290). The striking changes in the alpha-2-globulin in tuberculosis have been variously thought to be associated with a specific antibody to a fraction of tuberculin (286, 289), with a sensitization of the host (286), and with tissue destruction (290). A protective antibody against tuberculosis has been identified in gamma-globulin. In sarcoidosis, the hyperproteinemia is frequently due to a large increase in gamma-globulin (74, 288, 290). The other globulins are usually more concentrated than normal, and the polysaccharide associated with the alpha-2-globulin is increased, though not so much as in tuberculosis (290). Leprosy may produce large increases in alpha- and gamma-globulin (288). In syphilis, a small increase in serum globulin fractions, especially gamma (53, 231), is observed. The antibody responsible for the Wassermann and Kahn tests for syphilis is concentrated in a fraction of gamma-globulin (52, 53, 57, 231), but material giving a positive Hinton test has been found in another fraction (52). Anticomplementary activity is a normal property of gamma-globulin, but is inhibited by other serum fractions (56, 235). The hyperglobulinemia of lymphopathia venereum involves all fractions (166).

The common allergic diseases produce little change in the distribution of blood proteins, although antibodies can be identified in the gamma-globulin fraction (232). In the group of diffuse diseases of mesenchymal tissue which partake both of infection and allergy, the globulins show well defined changes. In acute rheumatic fever, there is an increase in alpha-globulin, and probably a larger and more prolonged rise in gamma-globulin than would have occurred in an uncomplicated streptococcal infection (68, 166, 190, 272). Essentially the same changes are seen in rheumatoid arthritis, regressing toward normal in remission or recovery (175, 190, 246, 339, 373). The increased globulin observed regularly in disseminated lupus erythematosus is found in the gamma-fraction (40, 190). Erythema nodosum is accompanied by increased alpha- and gamma-globulin and fibrinogen (190).

The different stages of glomerular nephritis lead to characteristic changes in the plasma proteins. In acute nephritis, albumin is reduced even more than indicated by the conventional salting-out methods (182). Gamma-globulin is increased in both relative and actual concentration and may remain so for many weeks. Alpha- and beta-globulin concentrations are normal unless an active infection is present (182). The nephrotic stage is associated with a profound reduction in the serum albumin and in gamma-globulin, while the alpha- and beta-globulins are increased (166, 169, 179, 190). The pattern is virtually diagnostic of the disease. A considerable part of the increase in alpha- and beta-globulins is due to lipoids associated with these fractions, which contribute more to the refractive increment than to the nitrogen content (16, 169). These fractions also have an appreciable solubility at the high salt concentrations used to precipitate "globulin." These properties result in unusually large discrepancies

between conventional and electrophoretic analyses (179). The albumin of blood and urine in nephrosis differs from normal in its electrophoretic behavior at pH 4, presumably due to some change in formation, since there is no significant difference between the albumins of nephrotic serum and urine (178). In the terminal stage of glomerular nephritis, the plasma shows a moderate reduction of albumin, and usually some increase in the globulins and fibrinogen (179, 190).

The urinary proteins in nephritis are identical in mobility with the corresponding serum fractions (179). The relative proportions of the various fractions in the urine are determined by two factors, the composition of the plasma and the permeability of the injured nephrons. In the presence of a constant renal lesion, it is possible to modify the excretion of a fraction by changing its concentration in the plasma. A renal "clearance" of that fraction can be calculated which appears to be independent of plasma concentration and urine flow over a limited range (181, 182). Spontaneous changes in the plasma proteins are often reflected in the urinary proteins, which may also be affected by a changing renal lesion. In the different stages of nephritis, the permeability of the kidney varies, especially to albumin, which is most readily excreted in the nephrotic stage (169, 179, 181, 182, 190). Fibrinogen and the lipoprotein complexes are only slightly excreted in any stage of the disease (169, 179, 181, 190). Albumin and alpha-1-globulin thus dominate the urinary pattern in the early nephrotic stage, while the onset of the terminal phase may be heralded by an increased proportion of globulins in the urine (20). In amyloid nephrosis, the plasma may contain large amounts of gamma-globulin which appears in considerable concentration in the urine, together with the other proteins of the serum (179). Other types of proteinuria show less distinctive patterns.

Disease of the liver is associated with characteristic changes in the plasma protein. In advanced portal cirrhosis there is a profound reduction of the serum albumin and a large increase in gamma-globulin, while the alpha- and beta-globulins show smaller increases (88, 179, 180, 190). Injury to the liver due to arsenic may produce similar changes, but beta-globulin may be more prominent (88). Infiltration by metastatic carcinoma produces less striking changes, with more reduction of albumin and less increase in gamma-globulin (88). Infectious hepatitis (catarrhal jaundice) is associated with a reduction in albumin and an increase in gamma-globulin, often with smaller increases in alpha-2- and beta-globulin (88, 191, 208). Obstruction of the bile ducts resulting in jaundice produces little change in the protein pattern for a time, but later the albumin may fall and beta-globulin may increase, possibly as a result of secondary changes in the liver (88, 166, 384).

Recently, there has been some interest in the signs of apparent liver disease which occur in malaria and infectious mononucleosis (42, 96). The similarity of the protein patterns in many chronic infections and in liver disease suggests that liver dysfunction or injury may play a part in the production of these changes in infections, in addition to the more obvious increment of antibody to the gamma-fraction. The colloidal gold and cephalin flocculation tests for liver disease depend on an increase in the gamma-globulin fraction (85, 124, 208), as well as a

decreased concentration and stabilizing power of the albumin fraction (208). Similar changes are found in malaria (96) and may well be present in other infections. The sedimentation rate of human erythrocytes, often used as a measure of the activity of an infection or of necrosis of tissue, is not specific in its response to any single fraction of the globulins or fibrinogen (97, 190, 290, 301) and may also rise with the increased globulins of liver disease and of other non-infectious origins (190).

Several diseases of the blood cells and of their precursors are followed by changes in the plasma proteins. These changes are particularly difficult to evaluate, since certain secondary effects of the disease may play a rôle in the alterations of proteins. The functions of the hematopoietic systems in the formation of plasma globulins (69, 127, 189, 378, 379) may be disturbed, and the liver may be injured by anemia or infiltration. Fever and malnutrition are frequently encountered. Perhaps, for these reasons, the changes observed in leukemia (166), Hodgkin's disease (328), and aplastic anemia (166) are not distinctive. The proportions of the plasma proteins are not disturbed in hemophilia (147), but an anticoagulant and a lack of the normal coagulant have been found in the plasma proteins of hemophiliacs (147, 224). The isoagglutinins of normal blood and certain pathological hemagglutinins have been identified in characteristic protein fractions (253, 318).

In multiple myeloma, the electrophoretic pattern of the plasma protein may be distinctive and sometimes virtually diagnostic of this tumor. Ultracentrifugation may show changes even more unusual than those demonstrated by electrophoresis (130, 196, 242). One of several types of protein with the mobility of a beta- or gamma-globulin may be present in the plasma, sometimes in large amounts (19, 95, 130, 166, 190, 212). The changes in the plasma are quite different in individual cases, ranging from a normal pattern or a slight reduction in albumin to a striking hyperproteinemia due to the accumulation of the abnormal protein. The urine may contain Bence-Jones protein of a mobility similar to the abnormal component of plasma, or may be free of protein despite the presence of large amounts of the pathological component in the plasma, or may contain considerable amounts of Bence-Jones protein which does not appear in detectable amounts in the plasma. When the abnormal component has the mobility of a beta globulin, its characteristics in the blood and urine are similar; but when the component of the mobility of gamma-globulin appears in the plasma, the urinary protein is apparently different from the new component of the blood (212). There is some loss of the normal serum proteins in the urine in many cases.

A reduction in serum albumin accompanies many types of tumor. The alpha-2-globulin and an associated polysaccharide are increased in many cases of carcinoma (290). When the liver is involved, the gamma-fraction may become more prominent (88, 290). In a similar way, the changes in other tumors may be more characteristic of the physiological changes than of the tumor itself (328). Infectious tumors of animals may cause changes in the plasma proteins. In certain strains of rabbits infected with papilloma, the beta-globulin fraction and

an associated lipoid are increased (298, 340). Fowl with leucosis show an increase in an abnormal component of the gamma-fraction as early as three days after infection (275). Injection of heat-inactivated tumor produces a similar change which occurs later and is less striking.

Several diseases of the endocrine glands cause changes in the plasma proteins. A reduction in serum albumin occurs in diabetes (149), Addison's disease (194, 195), and both over and under function of the thyroid (148). Corrective treatment is followed by a return of the concentration of albumin toward normal. Desoxycorticosterone acetate may not be as effective as whole adrenal extract in the correction of the depression of albumin in Addison's disease (195). The albumin may remain low despite conventional therapy when certain complications of diabetes (149, 278) and hyperthyroidism (148) are present, leading to the suggestion of high-protein diets under these circumstances. The increase in beta-globulin in diabetes (149, 290) and hypothyroidism (148) may be related to elevated blood lipids. In man, the alpha-globulin is increased in hyperthyroidism and decreased in hypothyroidism (148). A decreased albumin and increased alpha- and beta-globulins are observed in pregnancy (144, 172). The important rôle of the pituitary and adrenal glands in the formation of plasma proteins has been discussed in connection with antibodies. The defect in the serum proteins caused by ablation of the hypophysis (158, 215) can be best remedied by the injection of pituitary adrenocorticotrophic hormone (160). Growth hormone may be quantitatively less effective (160). Removal of the thyroid gland in the rat (219) is followed by a reduction of serum albumin, but the change in alpha-globulin appears superficially to be opposite to the change in human hypothyroidism. It may be pertinent that the affinity of lipoids for the varicus fractions of serum varies from species to species (60, 218). The increased alpha-globulin in the rat might be the equivalent of the increased beta-globulin in human hypothyroidism.

The proteins of several body fluids have been studied in normal and pathological conditions. The lymph (244), serous effusions (32, 109, 180, 276, 288), joint fluids (111, 246), and cerebro-spinal fluid (123) resemble serum in their protein pattern, unless modified by a local inflammatory process. The colloidal gold reaction, used clinically to estimate the protein distribution in spinal fluid, is produced by gamma-globulin and inhibited by albumin (86, 124), as in the case of similar clinical tests on the blood. The proteins of milk (312, 313), seminal plasma (84, 267, 268), pancreatic juice (226), and the fluids of the eye (110) have their own distinctive patterns.

CONCLUSIONS

Electrophoresis offers a versatile and accurate method for the study, separation, and analysis of proteins. Its applications to some biological and medical problems have been described. The method has aided in the separation of biologically important substances from native mixtures.

Electrophoretic analysis gives a distinctive picture of the composition of the proteins of the body. Such analyses may reflect physiological and chemical

changes in the body. The proteins of the plasma show characteristic patterns in a number of abnormal states. Like many other chemical and physiological measurements in disease, electrophoretic analysis of the plasma can not be expected to make a specific, etiological diagnosis, except in the gradually vanishing instance when only one cause of the physiological disturbance is known. Much more information is needed for the fundamental interpretation of the abnormal changes in the plasma proteins.

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PROBLEMS IN INVERTEBRATE ELECTROPHYSIOLOGY

THEODORE HOLMES BULLOCK

Department of Zoology, University of California, Los Angeles

Many aspects of the electrophysiological study of invertebrate nervous systems have been covered in recent reviews. Thus, the useful articles of Prosser (121) and Welsh and Schallek (149) specifically deal with invertebrates while those of Bishop (16) and Grundfest (64) include much recent work on these forms. Conduction and excitability in nerve are well treated by Curtis and Cole (46, see also Hodgkin and Rushton, 71). Wiersma (153) has dealt with muscle physiology in invertebrates. The rôle of acetylcholine is discussed in Nachmansohn (100) and Gerard (56). Chemical excitation of peripheral nerve is reviewed by Brink, Bronk and Larrabee (25).

Recent advances in certain areas of investigation have not, however, come to general attention. The present paper undertakes to summarize the present state of knowledge in four of the important fields of application of electrophysiological methods: the comparative study of synaptic mechanisms, physiological neuronography—which has been applied especially to giant nerve fiber systems, the phenomenon of spontaneous central activity and the identification and analysis of sensory structures. In each case particular attention is directed to problems and opportunities for further investigation.

Junctional transmission. As a result of recent work, the subject of comparative synaptology promises particularly interesting contributions to general neurology. Whereas the advantages of lower forms for the study of conduction lie in the apparent uniformity of the mechanism, much of the advantage for synaptology lies in the diversity of the manifestation of a perhaps common fundamental physico-chemical process. The diversity appears to rest on quantitative differences in physiologic properties and on differences in anatomical arrangement at the junction.

Nerve nets. Although electrical recording technics have not yet been successfully applied to nerve nets, it appears appropriate to discuss briefly the status of our understanding of this important type of nervous organization. The most primitive nervous systems known, those of the coelenterates, have for long been regarded as asynaptic. In order to explain local responses or subtotal response of any kind it was necessary to assume decremental conduction (Parker, 105), a phenomenon for which no independent evidence was cited and which was never successfully applied to the explanation of diversified behavior patterns in higher coelenterates. In 1935, as a result of the first modern physiological analysis of these systems, Pantin (103) offered an alternative explanation depending on the existence of discontinuities in the nerve net which require facilitation by successive impulses at critical intervals. Such neuro-neural junctions appear to be absent or permanently facilitated in extensive regions of the net, the specific reactions to stimuli depending on differentiated properties of neuro-myal junc-

tions in different muscles. But neuro-neural junctions were found, physiologically, by the dependence on facilitation. Junctions which depend greatly on facilitation are probably widespread among invertebrates. Though good evidence is at hand only for anthozoa and crustacea (Pantin, 104; Wiersma, 153) suggestive evidence has led Pantin to include echinoderms and molluscs (104) and Botsford to add annelids (21). Pantin's picture accords with available facts and explains observed responses in a satisfactory way (Pantin, 104; Bullock, 29). Anatomical evidence is notoriously difficult to obtain and to rely on in this matter but the reports of discontinuity in the net (Bozler, 23; Woolard and Harpman, 158) are generally given greater weight today than those (96, 105) of continuity (66, 74, 33, 104, 121, 42).

The importance of the question of the existence of synapses in the nerve net rests on the fact that if they are there, the well-known diffuse conduction in the net forces the acceptance of unpolarized synapses, unless secondary assumptions of duplicate paths in every direction are to be made. The balance of evidence today, although certainly not ruling out continuity in certain parts of the nerve net, indicates that unpolarized synapses exist in the nerve nets of coelenterates and probably enteropneusts and other groups (Bullock, 27, 28, 33). Nerve net in this usage becomes a physiological term denoting a diffuse locally autonomous plexus conducting in all directions. In the present state of knowledge the entities classically called nerve nets must be characterized in these terms since the exact anatomical status is unknown. In any case, loose usage of the term to include simple plexuses of peripheral terminals of neurons whether the cyton is far away as in human skin (15, 91) or in the periphery as in insects, worms and possibly other forms (155) must be deplored, even if anastomoses between sensory fibers are found. Unless evidence is available that the plexus can mediate responses locally, autonomously, is thus a distributing as well as receiving path, and can conduct diffusely, it should not be called a nerve net.

It may be hoped that electrical recording technics will be applied to these nervous systems. When they are, crucial contributions to the question of their functional organization and to the general understanding of common denominators versus derived properties of nervous tissue may be expected.

Even more neglected by modern physiological technics are the synapses and neuro-myal junctions in the several phyla of animals possessing ganglionated nervous systems simpler in organization than those of articulates and molluscs. Considering the great advances in the evolution of nervous function between the lowest and the highest invertebrates it would seem that efforts to analyze the properties of nervous units in these intermediate forms would be worth while.

"Typical" synapses in arthropods. Until recent months the only definite central synapses in invertebrates which had received careful study by the technics of electrical recording were those in the crayfish preparation of Prosser (111, 114, 119) and the cockroach preparation of Pumphrey and Rawdon-Smith (125).

Prosser's preparation includes the first synapses in the proprioceptive and tactile pathways, in particular those between primary sensory cells in the tactile hairs of the tail and second order neurons in the last abdominal ganglion. A

special advantage of this situation is the possibility of stimulating separately each of many single presynaptic units, permitting quantitative study of spatial summation, which in these junctions, in contrast to several others among the invertebrates, is necessary for transmission. Although the pathways are relatively fast, synaptic delay is long (2–8ms.). Transmission cannot follow above ten per second but facilitation is exhibited. After-discharge of the post-synaptic units is not marked. The preparation lends itself to the study of effects of external agents and a variety of drugs and salts has been tested (119). The intimate anatomy is still unknown.

The synapse in the last abdominal ganglion of the cockroach between primary vibration receptor afferents from the cercal nerve and large ascending second order neurons (125) presents an interesting contrast. Spatial summation is believed not to be necessary for the lowest threshold synapses in the rested state, but for all others (there are 12–16 second order fibers) supraminimal presynaptic volleys are necessary. Delay is as short as that in any natural junction known, including central synapses of mammals (but excluding the giant fiber septa of annelids and crustacea, whose status is still uncertain; see below), i.e., 0.6 ms. (Roeder et al., 133). The delay is much longer with weaker afferent volleys. Transmission can follow up to 50/sec. at high intensities of presynaptic stimulation, lower frequencies under submaximal stimulation, without showing any effects of the successive impulses. At higher frequencies some post-fibers drop out and delay is increased, although the remaining fibers continue to transmit in a one-to-one manner. In this semi-adapted condition facilitation occurs, as shown by recruitment of units upon shortening the interval between stimuli. After-discharge can be elicited (133). Lowenstein (93) has used time of abolition of response to air-puff stimulation in a method of bioassay of pyrethrum extracts, but it has not been established where, between receptor and second order axon, the compound acts. Roeder et al. (133) have used this preparation to study the effects of DFP, apparently the first test of this important anticholinesterase on single synapses. He reports a high sensitivity with increase in excitability and after-discharge as the first signs of effect. Later the synapses alternate between periods of complete block and periods of hyperactivity. Recovery from the DFP effect was not complete under the conditions used, but experience with this compound in other cases suggests that more acute experiments, using higher concentrations for shorter periods, may produce more marked effects with complete reversibility (40).

The properties of these synapses correspond in general to the usual textbook description and there is no reason to doubt at present that they are representative of most synapses in invertebrates. There exist, however, certain exceptional neurons in some of the higher invertebrates which display junctions of such character that their status as synapses is even uncertain. Special interest attaches to these cases and we may therefore turn to a brief review of the problem.

Annelid giant fiber discontinuities. Stough in 1926 (143) described and illustrated conspicuous septa across each of the three well-known giant nerve fibers in the dorsum of the earthworm nerve cord. The septa were shown to be com-

plete, oblique, simple partitions at segmental intervals. The unit of giant fiber between each two septa has one or several appended cell bodies so that the giant system can be thought of as a chain of compound neurons. Stough applied the term macrosynapse to these large, simple geometrically unpolarized junctions. Eccles, Granit and Young (49) showed by electrical recording that they conduct equally well in both directions and at a speed which permits essentially no time to be ascribed to delay at each junction. This has been confirmed (Rushton, 139, 136, 138; Bullock, 31, 32). As yet no physiologic sign of the septa has been reported although it is to be anticipated that such conspicuous discontinuity and differentiation of the fibers will be found to leave some mark in the conduction properties.

The question whether they must be regarded as synapses depends in the first place on the reality of the physiologic membrane and in the second upon the definition of synapse. Experimental demonstration of a functional membrane has not been made. But the considerable thickness of the histological membrane (143), the differentiation and interruption of neurofibrils (22, 142, 102, 32), and the presence of a clearly distinguishable doubly refractile layer continuous with the myelin around the fiber (144) suggest that the barrier is real. If this proves to be true the usual definition of a synapse as a region of functional contact between two discrete neuronal units will apply and the properties of polarization and delay common to most synapses could not be regarded as defining qualities. Instead they might be regarded as the results of a special anatomical arrangement (McCulloch, 97). The question may be met by adopting a more restrictive definition of synapse as is done by Bishop (16) who implies that a functional connection between discrete units cannot be regarded as a synapse until it has been shown to act as a switch which under natural conditions is not always closed. The utility of this criterion in specific cases may be questioned.

Reasons for doubting the reality of the membranes at the septa have been presented by Schoepfle (141). The argument is that the potential detected in the conducting medium around a nerve or muscle as a propagated wave of surface negativity approaches, is initially positive and immediately beyond the end of such a linear element is not followed by any negativity. Hence only an anelectrotonic or depressing effect could be exerted on another element whose excitable part was confined to this region of the external field of the prefiber, i.e., end-to-end junctions cannot be expected to transmit excitation but only inhibition. The argument is cogent if the geometry of the actual case corresponds to that of the theoretical one treated. A rigid analysis of the volume conductor problems involved with special reference to the expected effects of the oblique contacts (overlapping and tapering), in this case and other more complex forms found in other natural junctions is much needed for the proper evaluation of Schoepfle's argument and for the extension to actual situations of the schemes upon which Eccles has recently based a theory of synaptic transmission.

Eccles' theory (47, 48) also throws doubt on the reality of the septa of the earthworm as physiologic cell membranes. According to this concept a significant delay is theoretically necessary at any real junction, i.e., one where a mem-

brane like that around the axon interrupts the path of conduction. Apparently not all neurophysiologists are ready to accept that this is demanded by the membrane theory for all possible junctions (personal communications), but in any case it makes more important the precise determination of the degree of physiological discontinuity represented by the septa of the earthworm giant fibers. Conceivably they may provide a crucial test of Eccles' statement. The significance in this connection of the extremely short delays, often 0.1 ms., at the artificial synapses formed by the cut end of nerves or central tracts (Barron, 12; Renshaw and Therman, 127; Granit and Skoglund, 61), appears not to have been discussed. They are not readily attributed to subterminal interaction, which would have to occur 10 to 20 mm. from the cut surface in order to allow 0.5 ms. for delay, but neither has such a site been conclusively eliminated. Proper experiments for a clear localization of the synapse would seem to be practicable.

The septal system in the earthworm is not an isolated case. Among polychaetes giant fibers with cell bodies distributed along the length of the cord are common (90, 65, 54, 10). Septa have been described in only one form (143) but there have been no studies in which such reports might be expected since the first discovery of septa by Stough. It seems likely that many of the giant systems with distributed cell bodies will prove to have septa. One case is of special interest since the available anatomical information describes a typical synapse in each segment, with branching terminations of one fiber ending on the main stem of another (medial giant fiber pair of *Nereis*, Hamaker, 65). Yet conduction in these fibers and in all others tested in a recent study (15 species) is unpolarized and delays can be at most very short (35, 38).

The lateral giant fibers of the crayfish (85) and prawn (72) have definite septa and conduct both ways (Wiersma, 152; Prosser, 121). According to Wiersma (154) not more than 0.1 ms. can be attributed to delay at each.

A special problem is presented by the junctions between these lateral giant fibers. Both in crayfish (Wiersma, 154) and earthworm (Rushton, 136) a considerable delay (0.8 ms.) is introduced when an impulse crosses from one lateral to the other. There appears no reason to suppose the junction is polarized but it is easily fatigued in the crayfish. Rushton finds that in the earthworm impulses in the laterals which are thus made out of phase by 0.8 ms. somehow get into step if a sufficient length of fiber is available. Can electrotonic interaction of the type of Katz and Schmitt (87) be invoked—slowing down the leading or speeding up the trailing impulse?

Artificial synapses. Recently increased significance has been placed on studies of so-called artificial synapses. On the one hand they have been the only preparations available in which the properties of single junctions could be readily studied until Kuffler obtained isolated nerve muscle junctions (88). On the other hand they offer the valuable opportunity of varying the geometrical relations at the junction. These advantages have been best exploited in the work of Arvanitaki (8) on "ephapses" of squid giant fibers. She demonstrated that the pre-fiber could leave the post-fiber in an excited state only when it was arranged spatially in such a way that the impulse was not conducted past the

junction, thus permitting anelectrotonic depression to terminate the action. When the impulse in the pre-fiber stops soon after reaching the junction its final action is catelectrotonic and excitation takes place. The behavior of the preparations argues strongly for a purely electrical transmitter action and accounts quantitatively for the observed delay. There would appear to be no reason why an appropriate geometric arrangement of the elements could not provide an ephapse conducting in both directions, although this point was not tested. The properties of this preparation were particularly important in the elaboration of Eccles' (47, 48) picture of synaptic transmission.

Other artificial synapses which have been studied include the juxtaposed fibers of crab nerve (Arvanitaki, 7; Jasper and Monnier, 84; Katz and Schmitt, 87), and vertebrate nerve prepared in various ways to measure the effects of active fibers upon inactive ones (Marazzi and Lorente de No, 95; Bishop, 14). A preparation of special interest is the synapse formed at the cut end of nerve where impulses in fibers from the ventral root may be transmitted to fibers of the dorsal root (Granit and Skoglund, 61) to a degree depending on a complex of factors and with an apparent delay as short as 0.1 ms. It may be expected that much of significance will be learned as this type of preparation is studied with respect to the local processes and to comparison of nerves with component fibers of diverse proportions, excitability, accommodation and other properties.

Phenomena with probably the same basis have been observed in the central nervous system at the site of an interruption of the dorsal columns (12, 127) of the spinal cord. A very short delay is present in these cases also.

Artificial synapses in general have still much to offer, especially for quantitative study of those factors in transmission which are peculiarly under the control of the investigator in such preparations. They will continue to reward study even though natural synapses permitting observation of the unit junctions are rapidly becoming available.

Preparations for study of unit junctions. One of the most interesting results of recent work on various invertebrates has been the more or less incidental discovery of preparations permitting, with varying degrees of ease, the recording of activity of single synapses. The following are now available for this purpose. 1. The junction of primary sensory neurons from tactile receptors and second order ascending neurons in the last abdominal ganglion of the crayfish. 2. The similar synapse between cercal nerve afferents and giant central fibers in the last abdominal ganglion of the cockroach. 3. The synapses between medial as well as lateral giant fibers and segmental giant motor fibers in each abdominal ganglion of the crayfish. 4. That between the second order and third order giant fibers in the stellate ganglion of the squid. 5. Single nerve-muscle junctions isolated by dissection. It will be worthwhile to note the possibilities and limitations of these preparations.

1. Prosser's preparation has been mentioned above. While well suited to measurements of overall transmission properties and to experiments on altered external conditions, this preparation will probably not be convenient for recording the local signs of activity at the junction. Simultaneous activation of several

different ganglionic elements by each incoming impulse usually occurs, even though but one of these may be detected in the cord beyond the ganglion. Spontaneous firing of cells in the ganglion is continually going on under good conditions.

2. Pumphrey and Rawdon-Smith's preparation in the cockroach has many of the same advantages and disadvantages. The post-fibers being giant it may prove possible however to record local potentials relatively uncomplicated by small fiber activity, although the possibility of local reflex response (not appearing in the connective anteriorly) has not apparently been excluded. The existence of through fibers which are activated along with those that stop in the ganglion may prove a complication (but see 133). The principal limitations, however, are the difficulty of stimulating single pre-ganglionic units and the difficulty of confining the post-synaptic response to the same single unit for the duration of an experiment. There are a number of giant fibers, recruitment is well developed and the response at supraliminal stimulation is irregular.

3. Of quite a different character are the synapses in the crayfish between penultimate and final motor neurons of the giant system. These have been shown by Wiersma (154) to offer unique advantages. One asset, not yet fully exploited, is that the anatomy of the synapse, relative to most non-giant synapses, is quite accessible. It has been described for the related form, *Leander* (Holmes 72; see also Johnson, 85, on *Cambarus*). The four pre-fibers can be activated separately or in any combination, and in the root of the third peripheral nerve from each ganglion activity of a single post-fiber may be recorded. Some of the summation properties, the delay (0.7–1.0 ms.) and the fact of irreciprocal transmission have been reported but the possibilities for local response recording have not been explored. The circumstances are not ideal for single unit analysis owing to the presence of other junctions between the same pre-fibers and motor fibers of the first and second nerves.

4. The giant synapse in the stellate ganglion of the squid comes closer to the ideal for unit junction analysis. The intimate anatomy has been elegantly worked out by Young (162). A preliminary report of the physiologic properties has been published (Bullock, 37). There are two giant pre-fibers, whose synapses upon the post-fiber differ, and it is easy to stimulate one of these alone, although the other has not yet been so stimulated. A single post-fiber occurs in each of the stellar nerves and both pre- and post-fibers are of large diameter right up to the synapse. The fibers are in contact for nearly a millimeter. Leading directly from the ganglion, the local synaptic potential as well as both pre- and post-spikes are readily recorded.

Summation is not necessary; every presynaptic impulse is transmitted up to at least 300 per second. The delay is 1.2 ms. in a fresh preparation but can be doubled by fatigue. Transmission is polarized. Fatigue is readily brought on and maintained by stimulation at moderate frequencies, permitting study of the local response alone, while recovery and return of the spike can be induced at any time by reducing the frequency. Untransmitted impulses are thus seen to continue to exert an effect, preserving fatigue of the junction, apparently by

acting on the postsynaptic unit. Under such fatigue facilitation can be demonstrated. Refractory period of the local response may be absolute for a millisecond after which a response occurs but with an amplitude proportional to the interval, up to 2 or 3 ms. or more.[1] The synaptic potential when not intentionally depressed is estimated to rise at least three times higher than the threshold value for initiation of the propagated impulse. Its latency is not necessarily increased with fatigue but the height is reduced. The general picture forms an interesting comparison and contrast with the well-known work of Eccles (see 48 for references) on the synaptic potential recorded from many semi-synchronized synapses in the mammalian sympathetic ganglion. It is also very reminiscent of the artificial synapse of Arvanitaki (8) and the single neuro-myal junction of Kuffler (88).

5. The well-known work of Pantin (104 et ante) and Wiersma and van Harreveld (153 et ante) on the remarkable nerve-muscle relations in crustacea is being extended by study of the local events at the junction (Kuffler and Katz, 89, 86). Although it has apparently not been possible to isolate a single junction as was done in frog muscle, these authors are able to show the basis for the great dependence on facilitation. A single impulse arriving over an excitatory nerve fiber elicits only a small, non-propagated response comparable to the endplate potential described earlier in vertebrates. This local response grows with successive impulses at suitable frequencies (facilitation) and mechanical contraction accompanies it. Thus non-propagated, graded mechanical contraction is clearly demonstrated, in essentially intact limbs, as a normal mechanism, just as Wiersma (153) had predicted. Above a certain threshold of frequency propagated responses are said to occur. Confirming Wiersma and van Harreveld it is shown that impulses arriving over inhibitory nerve fibers can abolish both contraction and local potential produced by excitatory impulses. How this occurs can not yet be surmised but that it occurs subsequent to the action of the transmitter on the post-junctional membrane is indicated by the fact that facilitation continues during a period of inhibition, the response when inhibitor activity ceases immediately rising to the degree expected if no such period had supervened. The results do not offer any obvious aid in choosing between electrical and chemical transmitters, being compatible with either assumption. Further observations of this kind, particularly extensions of the technic to other muscles and other invertebrate groups will certainly repay effort.

Physiological neuronography. Dusser de Barenne (98) applied this term to the technic of mapping neuronal connections by following strychnine-induced spikes through the brain. It is equally appropriate for any technic designed to reveal intimate anatomy or functional organization by tracing electrical signs of nervous activity. This application of the methods of electrophysiology represents one of the most fruitful directions of investigation available to the comparative neurologist. Although well developed in vertebrate neurology, it has only begun to be exploited among invertebrates.

[1] In fresher preparations refractoriness of the local response may be absent at all intervals down to 0.2 ms., the shortest tested. Summation becomes prominent in these cases.

Prosser's (111) analysis of the pathways open to afferent volleys initiated in various parts of the crayfish is an excellent example of this approach, showing as it does many facts about functional neuroanatomy which would be impossible or extremely difficult to demonstrate by histological methods. Thus it was possible to specify that certain kinds of impulses, traveling in a given direction and resulting from a given type of stimulation, cross in the cord and others do not. The proper evaluation of the relations found largely awaits similar studies on related forms. The same author (113) mapped the area innervated by peripheral nerves in the earthworm, characterized the impulses elicited by various forms of stimulation and obtained evidence against the existence of a nerve net in the skin. Pringle (108) analyzed in a very successful way the reflex mechanism of the insect leg.

But virtually all other effort in this direction has concerned itself with the giant fiber systems found in widespread groups of both invertebrates and vertebrates. This is largely due to their technical accessibility, especially to electrical recording apparatus. The action potential in these fibers is so large that impulses in single fibers are easily recorded without elaborate dissection, indeed, in many cases from the external surface of the intact animal (139, 32, 35, 38). By tracing these impulses it is possible to reveal anatomical information such as the number of independent units, their extent (whether through-conduction or only short pathways), their anastomoses, contribution to peripheral nerves, connections with sensory and motor neurons and junctional properties.

Annelida. The first giant system from which action potentials were recorded was that in the earthworm. Eccles, Granit and Young (49), in a short note, reported that impulses were conducted in both directions and at a rather high speed. Earlier workers had concluded that the median fiber conducted posteriorly and the laterals anteriorly. Rushton (136, 138) and Bullock (31, 32) confirmed and extended these newer results. It was pointed out that the segmental septa which Stough (143) had shown to divide the fibers completely almost ten times every centimeter must not delay the impulse significantly. The problem of interpreting the septa is discussed above under "Junctional transmission." The earlier observations suggesting polarized conduction were explained by finding a topographic limitation of sensory connections to the giant fibers. The anatomically reported anastomosis between lateral fibers could be confirmed electrically. Motor connections have not been worked out but it is indicated that the three central giant fibers are probably not themselves efferent, that they elicit different muscular responses comprising the startle or withdrawal reflexes to stimuli applied to different parts of the body, and that the boundary between these regions must shift under different physiologic conditions (137).

Amassian and Floyd (5) describe repetitive firing of these fibers under certain conditions of galvanic stimulation.

The general features of the giant system are quite uniform among various oligochaetes but old anatomical evidence suggests that in the polychaetes a diversity of plan is to be found. This has begun to be exploited in recent studies (Bullock, 35, 38; Nicol and Young, 101). Many families of this large and diversi-

fied group lack giant fibers while others possess apparently fixed patterns consisting of from one unit to several dozen units. These may be compound or the process of a single cell, entirely central or efferent, long through-conduction paths or short, independent units, connected to sensory neurons throughout the body or only in restricted regions. All the through-conduction central fibers conduct both ways and agree in mediating a withdrawal response to startle stimuli. Giant fibers occur from less than 10 to more than 750 micra in diameter; apparent overall conduction rates, assuming no delays, fall in the range 1–10 m/s.

Arthropoda. Giant fiber systems in crustaceans, anatomically known from the accounts of Johnson (85), Lowe (92), and Holmes (72), have also been investigated physiologically only recently. Wiersma (152) reported unpolarized conduction and a certain pattern of interrelations between medial and lateral fibers as regards summation of effect on abdominal musculature in crayfish. Holmes, Pumphrey and Young (73) used these fibers in prawns to study conduction velocity as a function of fiber structure. Prosser and Altschule (121) report confirmation of Wiersma's general findings. The first extended account of a functional analysis of the crustacean giant system appeared in Wiersma's recent (154) report. He shows that the four distinct giant fibers all act on the same effectors, presumably through the same final motor neurons. No evidence is yet available of the difference in function between lateral and medial giants. Sensory connections are not known. As in the earthworm, the lateral fibers are connected together, but in the crayfish the connection is apparently synaptic, not anastomotic as has been assumed for the earthworm. Several interesting types of junctions between units of the system are discussed under "Junctional transmission."

The only studies on a comparable system in other arthropods are those of Pumphrey and Rawdon-Smith (125) and Roeder et al. (133) on the second order neurons in the auditory pathway from cercal receptors of the cockroach. These fibers ascend from the last abdominal ganglion, as through-conduction paths. Because of their high velocity and large diameter these authors use the term giant fibers but it may be questioned whether this is appropriate. In all other cases where the term is used a conspicuous discontinuity in size between the fibers in question and the next largest is recognizable. It is thus not used for the largest fibers in mammals though these are larger than some definite giant fibers in polychaetes, enteropneusts and others. Available evidence suggests that the large fibers in the cockroach as in other insects are the extremes of a continuous size spectrum. Nevertheless they permit recording and tracing of impulses and offer favorable opportunities for analysis of insect central nervous organization. The experiments of Pumphrey and Rawdon-Smith deal chiefly with the synapses in the last abdominal ganglion (see "Junctional transmission").

Mollusca. Until recently the only giant nerve fibers recognized in molluscs were those of decapods, although many early anatomists called attention to enormous nerve cells in various gastropods. Arvanitaki and Cardot have reported that the common snail *Helix* and the marine slug *Aplysia* display giant fibers in the interganglionic connectives (9, 41). Although an adequate descrip-

tive account of the anatomy is not given, a technic of extruding the cell bodies through their tube-like giant processes is presented. The chief interest of these authors has been the possibility of recording cell body potentials in isolated neuron somata. The potentials recorded in the connectives are remarkably long lasting (40 ms.) and slowly conducted (1 m/s). On present evidence it is difficult to orient the situation in these forms in terms of more familiar groups. Further elucidation of the gastropod plan of giant neurons will be awaited with interest.

The plan in decapods is at least as well known as that in any group, as a result of the anatomical work of Young (162). But, although the third order giant fiber from the squid has been the most studied giant preparation, as yet virtually no physiologic study has been made of the central organization of the system. The experiments of Young (161) on conduction through the stellate ganglion in the absence of cell bodies and of Bullock (37) on the properties of the synapse between second and third order giant fibers, in the same ganglion, are the only exceptions. The peripheral organization has been studied by Prosser and Young (123) and Pumphrey and Young (126) who have shown the neuromuscular relations and the significance of conduction velocity.

On the whole, the investigations made thus far upon giant fiber systems have been almost entirely exploratory. The preparations thus made available offer great opportunities for future work both upon functional organization at the level of comparative physiology and upon fundamental properties of units at the general physiologic level. Still other preparations are possible as well. Giant systems are known in many groups, even as low as the enteropneusts (30, 33) whose nervous system is very primitive. The high speed conduction tract in the mesenteries of anthozoans (*Calliactis*, Pantin, 103), overlapping as they do the lowest conduction rates observed in known giant fibers of polychaetes (38) suggest that possibly something very like a giant system is present already in the lowest phylum having a differentiated nervous system. Forms such as the stomatopod, *Squilla*, would seem to offer favorable circumstances for special studies, owing to large size and transparency of exoskeleton over the nerve cord; they have been found to possess good giant spikes (Bullock, unpub.). The need of further work on the giants of lower vertebrates (Mauthner and Müller fibers) may also be mentioned. These classical objects of anatomical study, source of some of our best cytologic knowledge of the vertebrate synapse, have only received their first electrophysiologic study (58). Of all giant systems least is known concerning the function in these vertebrate ones, much studied as they have been by experimental embryologic methods. It would seem that much of interest could be learned by recording from the fibers in life under conditions of physiologic, as well as direct, stimulation, but the necessity of finding particularly favorable species must be emphasized.

Spontaneous Central Activity. As is well known from the work of Adrian (1, 2), Rijlant (128), Prosser (109, 116), Bonnet and Bremer (19), Bonnet (17, 18), Roeder (134, 131), activity occurs in the central nervous system of arthropods in the absence of apparent stimulation. This has been extended to annelids (62, 63, 99, 34) and molluscs (26, 34) but as yet to no other groups besides verte-

brates. This last does not mean other groups have been found to lack such activity. Spontaneous activity has been found in every animal suitably tested. Like that in vertebrates, it is not fundamentally altered by deafferentation and persists in isolated ganglia or pieces thereof for long periods under conditions which need not be regarded as abnormal or excitatory. The consistent finding is striking that the general pattern of activity is much the same in the intact and probably excited animal, the intact and probably unexcited animal and the isolated ganglia after frank injury discharge ceases.

Interest in this type of nervous function has centered around certain problems: 1, spontaneity as a general phenomenon and its relation to rhythmic pacemakers; 2, the influence of environmental agents; 3, the bearing of such activity in invertebrates upon the interpretation of brain waves as seen in man, and 4, the significance of special types of pattern such as "visual rhythms."

Studies on the first of these have been thus far largely descriptive and have demonstrated the widespread occurrence of the phenomenon. It is present in the quiescent moth pupa (34) during a time when many of the neurons are dedifferentiating, others differentiating in a general reorganization of connections, sensory and motor functions are at a low ebb, appendages are absent and the musculature is largely dedifferentiated. It is apparent in small fragments of isolated ganglia, as in the cardiac ganglion of *Limulus* (6, 39). Even some peripheral sensory neurons exhibit spontaneous discharge (59, 94, 140, 53). But it is absent in many neurons under normal conditions; for example, giant neurons of earthworm, crayfish and squid. Experimental analysis has begun, with the study of agents which can alter the pace or initiate it in inactive cells and fibers (electrical polarization, 11, 50, 7, 39; oxidative control, 122; ions, 117, 118, 120, 131; temperature, 112; drugs, 20, 134; general, 50, 18, 24). Details cannot be given here but it is apparent that many factors contribute to the determination of frequency of a pacemaker and that under appropriate conditions any neuron can be made to discharge rhythmically in the presence of a steady state environment, in the manner of a relaxation oscillator. It is therefore a question of the individual properties of the neuron, its thresholds and time constants, whether and how active it will be under particular "normal" conditions. The phenomenon of spontaneity is, of course, not thereby explained but the quantitative definition of the difference between spontaneously active and inactive neurons is begun.

The relation of invertebrate manifestations of spontaneity to the interpretation of vertebrate brain waves has been discussed elsewhere (34). It was pointed out that comparative studies reveal a high degree of uniformity in the general pattern, especially the general position of the frequency spectrum of brain waves in all vertebrates. The uniformity is such that the smooth, slow (chiefly 1–30 per sec.) character of the activity obtains alike (though not without some significant differences) for intact human brain, deafferented cortex, isolated basal ganglia, the non-corticated frog brain and isolated fragments of the frog olfactory lobe as small as 0.1 mgm. "This, together with other evidence, suggests that brain waves should be looked upon not as a sign of the higher aspects of nervous activ-

ity but as a reflection of some basic, primitive common denominator of the brain of frogs and men." It is this general agreement in character among vertebrates that gives significance to the comparison with invertebrates, for a similar agreement exists among them, but the common pattern of invertebrates is strikingly different from that of vertebrates. The former are characterized by fast, spikey activity, many spikes having the time relations of single axon action currents and the dominant frequency being probably twenty times that of vertebrates. Discussion of the evidence leads to the conclusions that the prominence of spikes in one group and their virtual absence in the other is not readily attributed to size, or to complexity or synchronization, that slow waves comparable to vertebrate brain waves are present in many invertebrate ganglia (17, 18) and that both here and in vertebrate brain waves they are probably not explained as a resultant of impulse action currents but rather as a separate form of neuronal activity—a slowly oscillating change of state or beating (Gerard, 55) of masses of nerve cells in unison. In support of the last are such facts as these: the slow and spikey components in an insect brain can be altered by environmental agents separately, each can exist in the virtual absence of the other, in rhythmically discharging ganglia like the cardiac ganglion of *Limulus*, each discharge not only includes both components but the slow waves may initiate the discharge, preceding the first spike. Further evidence, including slow wave propagation in the absence of synaptic pathways, has been presented by Gerard (55) from studies on vertebrates and discussed in Bullock (34). The far-reaching implications of the assumption that neurons can affect each other by means distinct from classical impulses in synaptic pathways are obvious (see Bremer, 24). It seems apparent that the comparative approach to brain waves and spontaneous activity generally has significant contributions to make to the basic understanding of these aspects of neural function.

An important special case of slow activity has been extensively studied (Adrian, 3, 4; Crescitelli, 45; Jahn, Wulff, 80; Bernhard, 13) in the insect. Here rhythmic potentials occur which are not strictly spontaneous in that they are related to visual stimulation, even though they often occur for considerable periods after stimulation has ceased. Much work has gone into the descriptive analysis of the visual rhythms and the localization of the activity but as yet little work has been directed toward the problem of their basic nature. If their strong resemblance to vertebrate brain waves is more than superficial, as seems likely, they should represent useful material for experiments on the meaning of brain waves. Adrian (4) evidently regarded the oscillatory potential of the insect visual system as a composite of impulse discharges, but evidence has been cited (34 and below, p. 657) that although spikes may be synchronized with a certain phase of the slow waves the latter cannot be explained as an envelope or resultant of nothing but impulse spikes.

Sense Organs. As has been abundantly demonstrated in vertebrate physiology, one of the most successful applications of electrophysiologic methods is in the identification and characterization of sensory structures. Examples of this approach on invertebrate material serve to emphasize its possibilities. Although

some of these cases have been reviewed elsewhere, it seems desirable to bring them together with more recent ones to underline the opportunities for further work.

Prosser (110) discovered by action current recording the presence of a primitive photoreceptor system in the last abdominal ganglion of the crayfish. The properties of such cells are of great interest as they are apparently still undifferentiated anatomically from ordinary nerve cells (Welsh, 148). There are evidently only a few units but they can play a significant rôle in the activity of the animal deprived of eyes (148). Latency is long, maximum response is attained only after some seconds and may even occur after the light is turned off, adaptation occurs but slightly and slowly, activity continues for some seconds after the stimulus is removed. This preparation is well worth further attention.

Other primitive light receptors have been studied in the pelecypods. Hartline (68) found that one group of cells in the eye of the scallop responds, much like a large group of the elements of the vertebrate eye, with a burst of impulses at the "on," reaching highest frequency almost immediately (in contrast to the caudal photoreceptor of crayfish) and adapting greatly, ceasing promptly at "off"—with rarely an after discharge. Another group of cells also believed to be primary sense cells are "off"-responding like another large group of elements in the vertebrate eye—silent during illumination and firing at its cessation or reduction, to a degree dependent on the degree of illumination, suppressed promptly by reillumination, and often synchronized into rhythmic bursts. An especially notable feature of these two types of elements in the scallop eye is that they are anatomically separate and send their axons through separate nerves. Piéron and Segal (106) recorded slow waves and nerve action currents attributable to the scattered photoreceptors in the skin of the siphons of the clam *Mya*. On and off responding systems had distinct properties.

More advanced light receptors, forming well developed eyes, have been studied surprisingly little considering the elegance of the technics and the accessibility of the structures. Analysis of properties of units especially has lagged. The classical work of Hartline and his collaborators (57, 70, 69 et ante) on the single units in *Limulus* still stands unique. A point of special interest in one of the most recent contributions of this group (157) has not received the attention it deserves. Although off receptors are said to be absent from the *Limulus* eye, an off response is found in the ganglion where the primary sense cells synapse with second order neurons. The off response is as readily elicited by artificial stimulation of the nerve carrying the primary afferents as by photic stimulation of the eye: upon cessation of the afferent volley to the ganglion certain neurons therein discharge. This phenomenon has been heretofore associated with receptor elements only; the possibility that it may be a normal mechanism of central synaptic activation is not an accepted part of current neurophysiologic teaching. Studies directed toward the questions (a) is this in fact a generally used central excitatory mechanism? and (b) how can it be understood on any of the existing theories of synaptic transmission? are urgently needed.

A considerable body of work has accumulated dealing with the large slow po-

tentials associated with the illumination of photoreceptor elements in insects and cephalopods. These "retinal" potentials resemble in general the responses in vertebrate eyes and in some forms direct identification of waves with those of vertebrates appears possible (146). In other cases agreement has not been reached on the component waves even in closely related animals (13, 145). Analysis of the waves has led to the assignment of some to the optic ganglion and others to the receptors alone (80, 13). It has shown how the pattern varies with intensity and the state of adaptation (75, 43, 159, 67), with a remarkable diurnal rhythm in sensitivity (77, 79, 82, 159) and with temperature (81). In spite of the considerable effort, we are still much in need of comprehensive analytical work towards the definition of common denominators. And thus far the electrical events have not been successfully identified with any chemical or excitatory processes. Significant relations with nerve impulses are indicated, however, in the facts (1) that certain of the slow waves always begin before nerve fiber spikes appear, as is true in vertebrates (Adrian, 2; Bullock, unpublished observations), (2) at a certain phase of the slow "off" deflection all spike activity, even the spontaneous fraction, may be inhibited (Bernhard, 13) and (3) spike activity during illumination tends to be grouped into bursts in phase with the rhythmic oscillatory slow waves (4, 13, 36).

The distinction between two groups of fibers in the peripheral nerves of the earthworm, whose end organs are sensitive to two different kinds of stimulation (113), represents one of the most primitive cases of differentiated sensory structures physiologically recognized. It may be hoped that the method will be applied at even lower phyelogenetic levels. These findings doubtless represent the lowest known proprioceptors. Their properties and the possible occurrence of stretch receptors in other forms, including nemerteans and polychaetes, should be examined with special reference to the unique degree of extensibility, both active and passive, in these animals.

Proprioceptors have been identified elsewhere among invertebrates only in insects. As a result of the work of Pringle (107) special receptors in the cockroach can be designated as position sense organs though they are also receptive of other mechanical stimuli. They are of special interest because of their mechanism and properties and because of a peculiar importance in DDT poisoning. Pringle has recognized two kinds, campaniform sensilla and hair sensilla. Each is distributed in specific patterns on the appendages and body, strategically located to detect stresses in the exoskeleton and movement of folds in intersegmental membranes, respectively.

The campaniform sensilla appear to be specifically sensitive to compressive forces in the cuticle and each end organ, indeed each group of sensilla is oriented so as to detect only components along a certain axis. Their properties are similar to some types of vertebrate proprioceptors but a difference of probably broad significance is that they respond not to tension in individual muscles but to the integrated result of several muscles, the force of gravity and the position of the member in relation to the force. Here is yet another case of the peripheral integration seen in so many invertebrate sensory and motor relations. A point

of anatomical interest is the strong probability that the nerve fibers from several end organs join to form a single afferent fiber in the peripheral nerve—as is likely also in annelids where many more primary sense cells than fibers in the segmental nerves are found (113). It should be emphasized that there are probably many other receptors acting as proprioceptors, for the anatomy of insects is such that most mechano-receptors (tactile hairs, chordotonal organs, etc.) must respond to touch, pressure, muscle tension, gravity and movement, rarely being able to distinguish one form of stimulus from others (see Wigglesworth, 155).

Roeder (132) and Roeder and Weiant (135) have shown these structures to be especially susceptible to DDT and probably the first organs to respond to this poison, although no definite relation between its action on these receptors and its lethality can be stated. In great dilution DDT evokes prolonged trains of impulses in the nerve fibers from the proprioceptors, bombarding the central nervous system with an intensity that might well explain some of the observed motor effects (147).

Sound receptors have been studied electrically in insects by Wever et al. (151, 150) and Pumphrey and Rawdon-Smith (124), whose work is reviewed by Wigglesworth (155).

Among the more conspicuous opportunities in the area of sensory physiology the following may be mentioned: confirmation and characterization of the gyroscopic organs of equilibrium in the halteres of dipterans and others (Wigglesworth, 156); demonstration of the distinctness, or lack of it, of receptors for special forms of stimuli—thus, touch and pressure, pressure and muscle tension, heat and cold, nociceptive and heat or cold, etc. (Frings, 52), subdivisions of the chemical sense (51, 155); single unit analysis of photoreceptors for the direct demonstration of color reception in insects and others—in the manner of Granit's already classical work on vertebrates (see 60).

If any general conclusions are to be drawn from the group of studies here cited they would certainly emphasize anew the pregnancy of the comparative approach both for providing leads, clues and perspectives of value in strictly mammalian neurophysiology and for providing material uniquely suited to many fundamental experiments in general neurology. Thus far work in this area has gone little beyond exploration; the principal opportunities are still ahead.

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